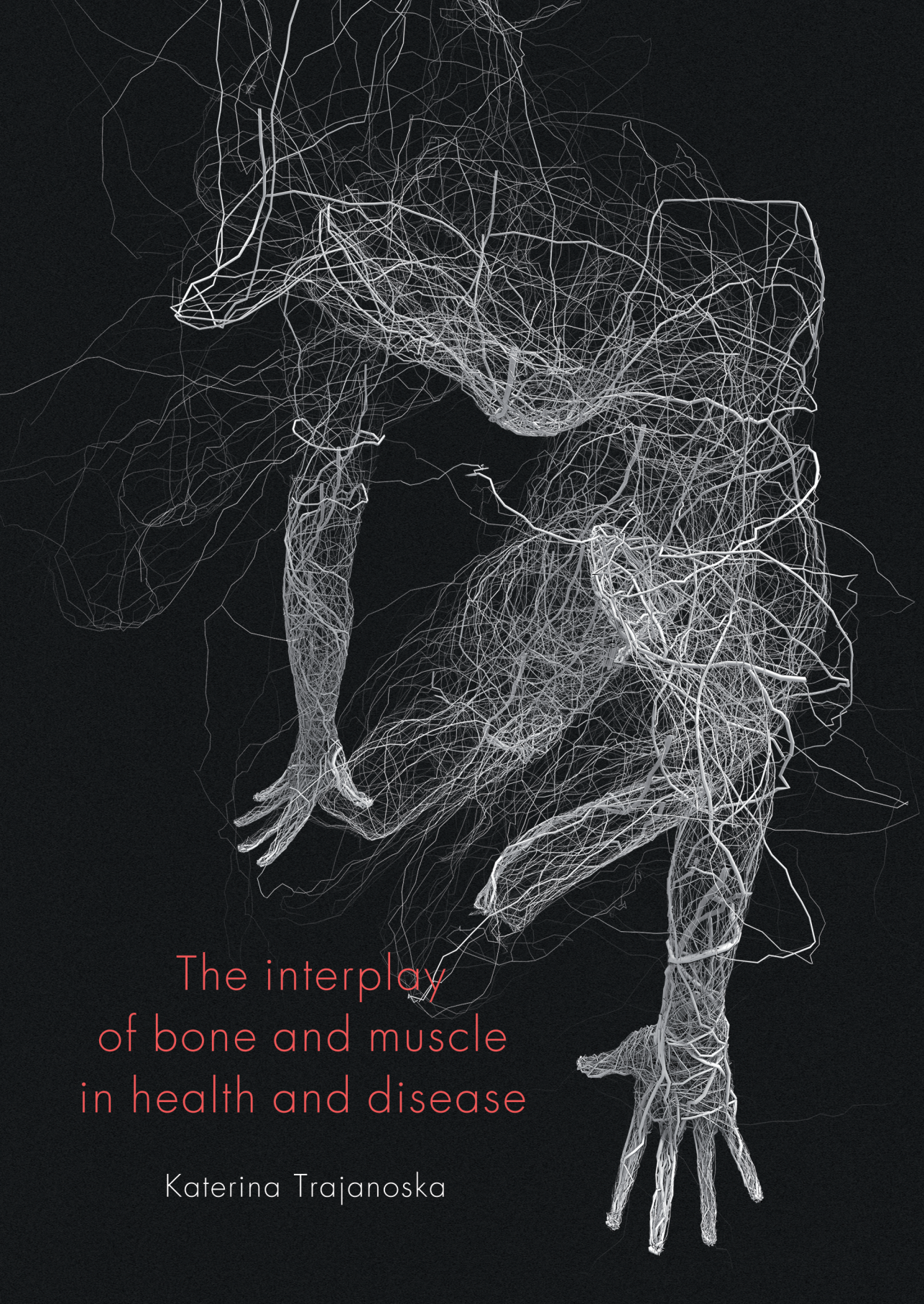


The interplay of bone and muscle in health and disease

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The Interplay of Bone and Muscle in Health and Disease

Het samenspel van botten en spieren bij gezondheid en ziekte

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. R.C.M.E Engels
and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Wednesday, 7 October 2020 at 9:30 hrs

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"*Have patience with everything that remains*
unsolved in your heart.
Try to love the questions themselves,
like locked rooms and like books
written in a foreign language
... live in the question."

Rainer Maria Rilke

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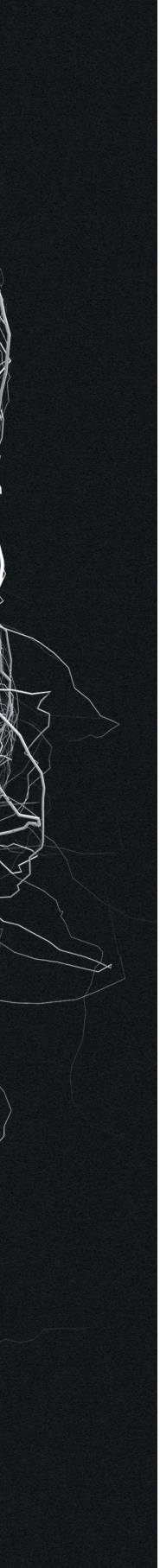
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1

General Introduction

MUSCULOSKELETAL AGING: OSTEOPOROSIS, SARCOPENIA, FALLS AND FRACTURES

"Aging is inevitable. Healthy aging is not"

Aging is a natural and inevitable part of our lives. Over the past decades, the average global life expectancy has been increasing at a rapid rate due to the decrease in all-cause mortality. This has resulted in significant growth of the fraction of older people in the population, especially among those 65 years and over, including centenarians (reaching an age of 100 years).[1] In the last five decades, the number of people aged 50 and older has quadrupled to more than 1.6 billion. Accordingly, the prevalence of chronic diseases has also increased, with more than 80% of the individuals above the age of 65 suffering at least one chronic condition.[2] A substantial proportion of this disease load is attributed to musculoskeletal disorders.[3] With aging the musculoskeletal tissues function less effectively due to changes in their quantity and quality. [4] Several pathological mechanisms can explain the tissue alterations with aging such as imbalance in matrix synthesis and degradation, altered matrix composition and decline in the number of effective stem cells (**Box 1**).[4] When tissue damage accumulates and exceeds a certain threshold it becomes clinically evident and manifest across muscle, bone, cartilage, and tendons.

Box 1 | Mechanisms driving the age-related musculoskeletal tissue changes

- Decrease in the amount of tissue, usually secondary to an acquired imbalance in matrix synthesis and breakdown.
- Altered molecular composition of the matrix, particularly post-translational modification of structural proteins such as collagen and elastin.
- Accumulation of degraded molecules in the matrix.
- Reduced efficiency of functional tissue elements.
- Reduced synthetic capacity of differentiated cells.
- Decline in effective stem cell populations.
- Altered levels of circulating trophic hormones, growth factors and cytokines, or an altered ability of the cells to respond to them.
- Alterations in the loading patterns of the tissue or the tissue's response to loading.

Source: Freemont, A. & Hoyland, J. Morphology, mechanisms and pathology of musculoskeletal ageing. J. Pathol. 2007

Musculoskeletal disorders and their sequels are the most common cause of pain and physical decline in the elderly and are among the leading contributors of years lived with disability worldwide. [5] The decline in bone mass and quality i.e., **osteoporosis,** and muscle mass and strength i.e., **sarcopenia,** are highly prevalent in elderly people

and are the main focus of this thesis together with their sequels i.e., **falls and fractures**. Both falls and fractures represent a serious health issue leading to loss of confidence and independence, increased morbidity and mortality that together impose a large burden on the healthcare system, patients, their families and society in general. Thus, besides aiming to further extend the lifespan what is more relevant now is procuring to achieve a longer *healthy* lifespan. Identifying factors leading to sustainable aging free of morbidity e.g., musculoskeletal and co-morbidity is a pivotal step in the realization of the ideal of healthy aging.

"...years are being added to our lives, life is not being added to our years: the extra years are being added at the very end of our lives and are of poor quality" [6]

Guy C Brown

CONTEMPORARY DEFINITIONS OF OSTEOPOROSIS AND SARCOPENIA

Osteoporosis, derived from the Greek terms for ***bone and pore***, was coined in the mid-1830s to describe porous bones. Many definitions of osteoporosis have followed and in the early 1990s WHO issued a consensus statement which defined osteoporosis as a systemic disease characterized by ***low bone mass and microarchitectural deterioration of the bone tissue leading to increased fracture susceptibility***. From this definition, clinical cut-off points were established for bone mineral density (BMD) measured by Dual-energy X-Ray Attenuation (DXA) at the femoral neck or the lumbar spine (the most prominent skeletal sites of fracture). A diagnosis of *osteoporosis* conforms to BMD levels lower than -2.5 SD below the reference for young adults, while BMD levels between -1 and -2.5 constitute *osteopenia*. [7] This classification has been widely adopted in clinical practice and -very importantly- to define treatment indication. While DXA-based BMD provides a quantifiable assessment of fracture risk, it comes short to assess properties of bone mass distribution and microarchitecture. Hence, other assessments like Trabecular Bone Structure (TBS) [8] determined from DXA images have been developed. Further, as DXA measurements are two-dimensional and only provide a measure of areal BMD, additional methods such as peripheral Quantitative Computerized Tomography (pQCT) have evolved to provide additional information regarding bone volume and/or microarchitecture for which clinically relevant thresholds are yet to be defined (**Figure 1**).

Sarcopenia, derived from the Greek phrase ***poverty of the flesh***, was defined for the first time in the late 1980s as an age-associated loss of lean mass. [9] However, since with aging two additional aspects of the muscle are changing i.e. strength and

performance, the definition of sarcopenia has been evolving over the years supported by collaborative efforts from several research groups around the world. [10,11,12,13] Currently, all distinct consensus definitions characterize sarcopenia by the presence of both ***low muscle mass and reduced muscle function (strength or performance)***. There is a wide variety of diagnostic tests and tools available to determine sarcopenia (**Figure 1**). However, an agreement on the "*golden standard*" tool and cut-off points to define this condition, is still lacking. As consequence, the comparison of observational studies is challenging and the early diagnosis and intervention of sarcopenia can also be hampered. To facilitate the clinical diagnosis of sarcopenia, the European Working Group on Sarcopenia in Older People (EWGSOP) has provided recommendations for early detection of sarcopenia in clinical practice. [14] The biggest milestone was the recognition of sarcopenia as a clinical condition in 2016 (ICD-10-CM M62.84). [15] Sarcopenia is clinically relevant syndrome as it is associated with variety of adverse health outcomes, such as disability, falls and increased mortality. Therefore, it is important to raise the awareness about sarcopenia among the medical professionals and the general population.

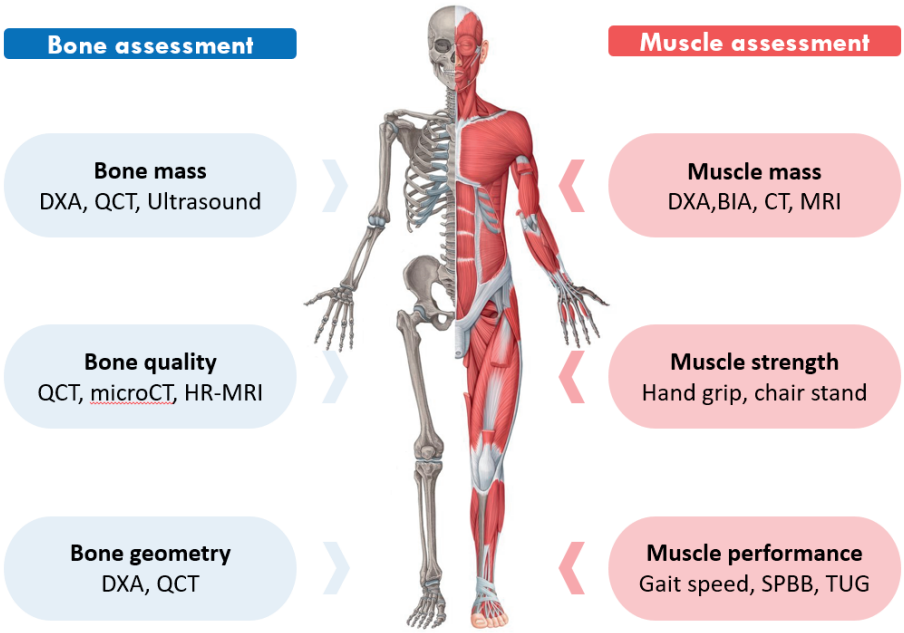


Figure 1 | Non-invasive techniques for assessing bone and muscle mass and quality.
DXA= dual-energy X-ray absorptiometry; BIA= bioelectrical impedance analysis; QCT= quantitative computed tomography; MRI= magnetic resonance imaging; HR= hight resolution; SPBB= short physical performance battery; TUG= timed up and go.

CONSEQUENCES OF OSTEOPOROSIS AND SARCOPENIA: FRACTURES AND FALLS

Each year, almost a third of all people aged 65 and over fall at least once and there are an estimated 8.9 million new fragility fractures worldwide. [16] Fragility fractures are fractures that result from mechanical forces that would not ordinarily result in fracture, i.e., low energy trauma.[17] They are highly prevalent in individuals over 50 where approximately one in two women and one in four men will experience at least one major osteoporotic fracture (hip, wrist, humerus or clinical vertebral fracture). The age-related decline in BMD is the strongest non-modifiable risk factor for fracture. Besides age many other modifiable risk factors can lead to low BMD and significantly increase the fracture risk; thus, early identification of people at higher risk of developing osteoporosis, and subsequent intervention, can have tremendous effects in fracture prevention. Muscle mass is an important determinant of BMD. It can determine the rate of both bone accrual in children [18] and bone loss in older adults. [19] Sarcopenia has been associated with increased fracture risk, however, these effects have been mainly driven by low BMD. [20,21] Nevertheless, sarcopenia can lead to fracture by pathways independent of BMD such as risk of falling. Indeed, sarcopenia is one of the many modifiable risk factors for falls [22] and the risk of falling appears to increase with the number of risk factors such as changes in vision, balance, sedentary lifestyle and medication use. [23] Falls are a relevant predictor of fracture causing almost 90% of all hip fractures [24] and within the first six months after hip fracture, up to half of the people fall again. [25] Prevention strategies focused on improving muscle and bone health will substantially decrease the falls and fracture incidence and reduce the associated physical and economic burden in the following years.

DETERMINANTS OF OSTEOPOROSIS AND SARCOPENIA: WHEN NURTURE AND NATURE COLLIDE

"The problem, of course, with the idea of nature versus nurture was that it posed a choice between determinisms." *James S.A. Corey*

Variety of **genetic, epigenetic, mechanical, biochemical, and lifestyle factors** determine the growth, accrual, maintenance and, loss of bone and or muscle mass through the life-course. **Common risk factors** for osteoporosis and sarcopenia include age, sex, sedentary lifestyle, poor diet, smoking, alcohol consumption, corticosteroids, low vitamin D, co-pathology [26,27] (**Figure 2**). In addition to these risk factors, osteoporosis and sarcopenia are interconnected with the constant mechanical and biochemical cross-talk within the bone-muscle unit [28]. For instance, bone properties are regulated

by **mechanical forces** at different levels, as demonstrated by the various developmental and functional aberrations that arise in the absence of muscle stimuli or loadings [29]. In this fashion, the loss of muscle mass and strength in older adults will have impact on the rate of bone loss as well. Furthermore, a variety of anabolic or catabolic molecules released by skeletal muscle (e.g. myokines, IFG1, FGF2) [30] or by bone (e.g. osteocalcin, sclerostin, IGF-1) [31,32] may have beneficial or detrimental effects on each other (**Figure 2**).

The effects of the environment are important; however, our phenotype is also in part shaped by variation in our **genome** (DNA). Some phenotypes such as the colour of our eyes or hair are largely determined by genetics. Similarly, monogenic disorders, i.e., Mendelian diseases, are strongly influenced by variation in one single gene. On the other hand, sarcopenia and osteoporosis are complex disorders which are result of the joint effect of numerous variations across the genome and their interaction with environmental factors. The most common genetic variation is the **single nucleotide polymorphism (SNP)** defined as a variation in a single DNA base (A, C, G or T) which can be detected using existing SNP genotyping assays. With the advent of SNP genotyping microarray technology, it is now possible to genotype a proportion of SNPs

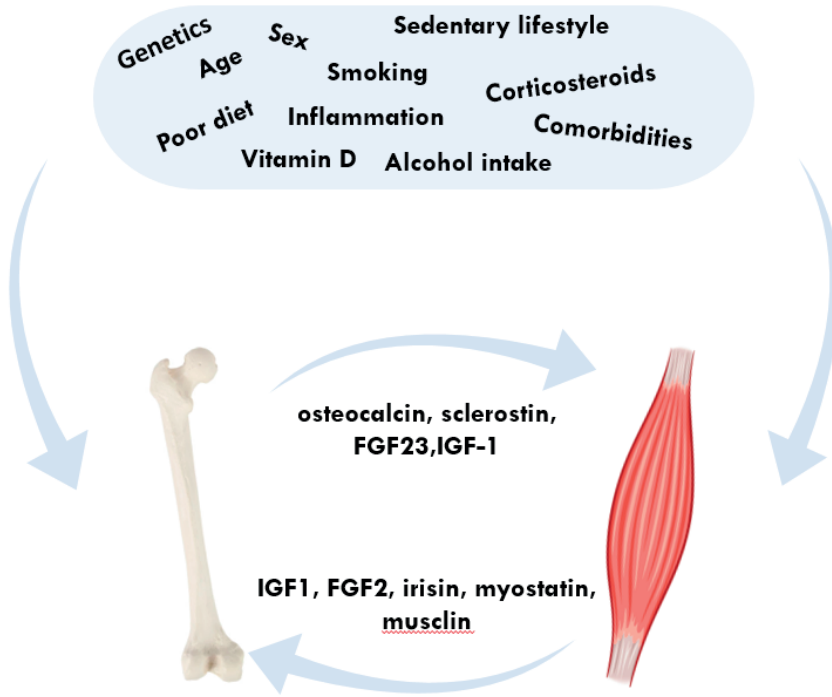


Figure 2 | Risk factors affecting both muscle and bone, and muscle-bone cross talk.
IGF= insulin growth factor; FGF= fibroblast growth factor.

(typically around 500,000), while the reminder (up to 300 000 000) are imputed using reference panels of individuals with dense genotype data such as HapMap, or the 1000 genome, HRC or TOPMed projects (among others). Using **genome-wide association studies (GWAS),** we can analyse these measured variations i.e. SNPs in relation to specific traits or diseases within a hypothesis-free approach (i.e., comprehensive genome scans without any previous knowledge on gene function). The effect sizes of the causal genetic variants and their corresponding frequencies are the determinants of the genetic architecture of a given trait or phenotype. For common diseases and complex traits, effects per SNP are typically small arising from common to less frequent variants. Hence, the power to detect associated genetic variants is highly driven by the sample size of a study (or meta-analyses thereof). The larger the sample size the higher the power to detect novel associated variations across the genome. In order to maximize the statistical power many collaborative efforts have been established across the world which have had unprecedented success, and the musculoskeletal field has been no exception (reviewed in **Chapter 3.2.1**[33] and **Chapter 4.1**[34]). These genomic discoveries have substantially expanded our knowledge of the gene-disease relationships.

Moreover, **epigenetic regulation** of bone and muscle metabolism have been shown to also play a role. Studies have shown, for instance, that microRNAs are associated with bone and muscle homeostasis [35,36]. Similarly, (total body) muscle mass and (total body) bone mass are highly genetically correlated (r~0.40)[37]; indicating that there is overlap between genetic influences on bone and the genetic influences on muscle. Significant genetic overlap across phenotypes can be due to pleiotropy, strictly speaking, when one gene controls the expression of multiple traits. To date, several genes have been proposed to affect both bone and muscle, such as *SREBF1, GLYAT* and *METTL21C* (reviewed in **Chapter 4.1**)[37,38,39]. There are several methods to investigate pleiotropy namely classified as univariate and bi-/multivariate. The latter methods require all phenotypes to be measured on the same individual, i.e. individual level data; whereas, univariate methods are based on GWAS summary statistics data of one trait. Identifying pleiotropic genes can be beneficial for the joint treatment of both sarcopenia and osteoporosis, i.e., osteosarcopenia, which often co-occur in elderly people.

"In the real world there is no nature vs. nurture argument, only an infinitely complex and moment-by-moment interaction between genetic and environmental effects." *Gabor Mate*

SCOPE OF THIS THESIS

The **aim** of this thesis is to identify genetic and environmental risk factors of osteoporosis, sarcopenia and their sequels i.e., falls and fractures. The epidemiological studies were embedded within the **Rotterdam Study (RS)** a prospective population-based study including inhabitants of the Ommoord district in the city of Rotterdam, the Netherlands and divided into three cohorts [40]. Participants underwent a wide range of periodic examinations in a research facility in the centre of their district, repeated every 3-4 years using and complemented with home interviews. The Genetic Laboratory of the Erasmus MC preformed the genotyping of all three RS cohorts. The study is approved by the medical ethics committee and all participants have provided written informed consent to participate in the research. Genetic studies of the RS are carried out within the setting of large collaborative efforts, namely the **GEnetic Factors for OSteoporosis (GEFOS)** and **Cohorts for Heart and Healthy Research in Genomic Epidemiology (CHARGE**) consortia. These are international networks involving various prominent research groups around the world with a general objective to identify the genetic determinants of musculoskeletal and aging-related traits [41]. In addition we made use of the **UK Biobank Study**, one of the largest genetic biobanks in the world [42].

Chapter 2 addresses the determinants of osteoporosis and fracture risk. In **Chapter 2.1.1,** we estimated the incident rates and trends of site-specific non-vertebral fractures in the Rotterdam Study over 20 years of follow-up and determine the predictive value of one single BMD measurement. **Chapter 2.1.2** is an observational study which explores the effects of metabolic syndrome and its components on different aspects of bone health. **Chapter 2.1.3** examines the association between osteocalcin, adiposity and bone health in elderly people using a cross-sectional study design. **Chapter 2.2.1** is a review paper summarizing the current state and evolution of the studies on the genetics of osteoporosis and fracture risk. **Chapter 2.2.2** is a GWAS study which aimed to disentangle the genetic background of circulating osteocalcin. **Chapter 2.2.3** provides insights into the genetics of total body BMD and examines possible age-specific effects using GWAS approach while **Chapter 2.2.4** explores the genetic and clinical determinants of risk of fracture within a GWAS and MR study design (**Box 2**). **Chapter 3.2.5** is a review paper of the causal inference (Mendelian randomization) analyses that have been performed in relationship with bone. Next, **Chapter 3** focuses on sarcopenia, considered a novel geriatric syndrome, and its risk factors and consequences. In **Chapter 3.1,** we estimated the phenotypic correlations between a variety of bone and muscle parameters derived from DXA and pQCT. In **Chapter 3.2,** we estimated the overall prevalence of sarcopenia in older adults and explored the most common clinical correlates of this pathology. In **Chapter 3.3,** we conducted a systematic review and meta-analysis to estimate the overall prevalence

of sarcopenia in patients with chronic obstructive pulmonary disease whereas in **Chapter 3.4** we evaluated the relationship between body composition and sarcopenia with non-alcoholic fatty liver disease in an observational study of elderly people. **Chapter 4** examines the pleiotropic effects in the musculoskeletal system **Chapter 4.1** is a review paper on the current knowledge of the bone and muscle interactions in humans. In **Chapter 4.2,** we used a bivariate genome-wide approach to search for possible pleotropic genes affecting bone and muscle whereas in **Chapter 4.3** we explored the genetic landscape of falling risk by preforming GWAS analysis and estimated the shared heritability of falls with bone and muscle phenotypes. In **Chapter 4.4** we examined the shared genetics between osteoarthritis and BMD using a systematic overlap analysis on a genome-wide scale. Finally, **Chapter 5** discusses the findings and provides suggestions for future research.

Box 2 | Glossary of Mendelian randomisation

Mendelian randomization (MR) is a statistical technique that leverages genetic information in order to provide evidence for a causal relationship between modifiable risk factors and diseases.

Natural experiment

The MR approach uses genetic variants as instrumental variables for the risk factor of interest. Due to the random allocation of alleles during gamete formation the genetic variants are less likely to be associated with any confounders. Importantly, they cannot be affected by reverse causation. Therefore, the MR approach can provide more robust evidence of causal associations compared to the traditional observational studies.

Key assumptions

1. The genetic variants must be associated with the risk factor under investigation.
2. The genetic variants are not associated with any confounder that can bias the association between the risk factor and the outcome.
3. The genetic variants affect the disease under investigation only through the risk factor of interest.

Study design

One sample MR – when both the risk factor and outcome are measured in the study population.

Two sample MR – when the risk factor and outcome are measured in two different study populations. This methodology has been facilitated by the advent of large-scale GWAS that have led to substantial increases in the statistical power of the MR approach.

Limitations

- Heterogeneity – Presence of differences in effects estimates between the genetic variants used as instrumental variables for the risk factor under investigation that cannot be explained by sampling variation alone.
- Population stratification – Presence of differences in allele frequencies and/or disease prevalence rates between subgroups in the total study population which can confound the association between the risk factor and the disease of interest.
- Pleiotropy – When one genetic variant is associated with more than one trait which is a serious violation of the third MR assumption.
- Canalization – When the individuals' response to genetic and environmental influences is attenuated or absent as a result of the presence of so-called "buffering mechanisms" that act against the expected genetic and environmental effects.
- Weak instruments – When the genetic variants explain a small proportion of the variation of the risk factors, MR can provide biased causal estimates due to very low statistical power.

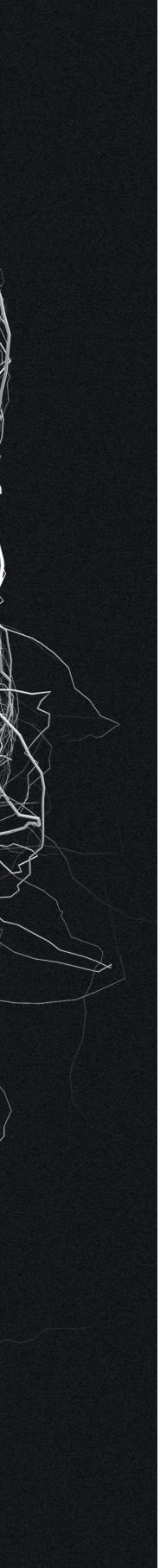
REFERENCES

1. Christensen, K., Doblhammer, G., Rau, R. & Vaupel, J. W. Ageing populations: the challenges ahead. Lancet (London, England) 374, 1196–208 (2009).
2. Fontana, L. Modulating human aging and age-associated diseases. Biochim. Biophys. Acta 1790, 1133–8 (2009).
3. Fejer, R. & Ruhe, A. What is the prevalence of musculoskeletal problems in the elderly population in developed countries? A systematic critical literature review. Chiropr. Man. Therap. 20, 31 (2012).
4. Freemont, A. & Hoyland, J. Morphology, mechanisms and pathology of musculoskeletal ageing. J. Pathol. 211, 252–259 (2007).
5. GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, S. L. et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet (London, England) 392, 1789–1858 (2018).
6. Brown, G. C. Living too long: the current focus of medical research on increasing the quantity, rather than the quality, of life is damaging our health and harming the economy. EMBO Rep. 16, 137–41 (2015).
7. Kanis, J. A. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. Osteoporos. Int. 4, 368–81 (1994).
8. Harvey, N. C. et al. Trabecular bone score (TBS) as a new complementary approach for osteoporosis evaluation in clinical practice. Bone 78, 216–224 (2015).
9. Rosenberg, I. H. Sarcopenia: Origins and Clinical Relevance. J. Nutr. 127, 990S-991S (1997).
10. Cruz-Jentoft, A. J. et al. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. Age Ageing 39, 412–423 (2010).
11. Fielding, R. A. et al. Sarcopenia: An Undiagnosed Condition in Older Adults. Current Consensus Definition: Prevalence, Etiology, and Consequences. International Working Group on Sarcopenia. J. Am. Med. Dir. Assoc. 12, 249–256 (2011).
12. Chen, L.-K. et al. Sarcopenia in Asia: Consensus Report of the Asian Working Group for Sarcopenia. J. Am. Med. Dir. Assoc. 15, 95–101 (2014).
13. Studenski, S. A. et al. The FNIH Sarcopenia Project: Rationale, Study Description, Conference Recommendations, and Final Estimates. Journals Gerontol. Ser. A 69, 547–558 (2014).
14. Cruz-Jentoft, A. J. et al. Sarcopenia: revised European consensus on definition and diagnosis. Age Ageing 48, 16–31 (2019).
15. Anker, S. D., Morley, J. E. & von Haehling, S. Welcome to the ICD-10 code for sarcopenia. J. Cachexia. Sarcopenia Muscle 7, 512–514 (2016).
16. Johnell, O. & Kanis, J. A. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos. Int. 17, 1726–1733 (2006).
17. Kanis, J. A. et al. The Burden of Osteoporotic Fractures: A Method for Setting Intervention Thresholds. Osteoporosis International 12, 417–427 (Springer-Verlag London Limited, 2001).

18. Greene, D. A. & Naughton, G. A. Adaptive Skeletal Responses to Mechanical Loading during Adolescence. Sport. Med. 36, 723–732 (2006).
19. Verschueren, S. et al. Sarcopenia and its relationship with bone mineral density in middle-aged and elderly European men. Osteoporos. Int. 24, 87–98 (2013).
20. Harvey, N. C. et al. Measures of Physical Performance and Muscle Strength as Predictors of Fracture Risk Independent of FRAX, Falls, and aBMD: A Meta-Analysis of the Osteoporotic Fractures in Men (MrOS) Study. J. Bone Miner. Res. 33, 2150–2157 (2018).
21. Buckinx, F. et al. Pitfalls in the measurement of muscle mass: a need for a reference standard. J. Cachexia. Sarcopenia Muscle 9, 269–278 (2018).
22. Landi, F. et al. Sarcopenia as a risk factor for falls in elderly individuals: Results from the ilSIRENTE study. Clin. Nutr. 31, 652–658 (2012).
23. Quality statement 2: Multifactorial risk assessment for older people at risk of falling | Falls in older people | Quality standards | NICE.
24. Hayes, W. C. et al. Etiology and prevention of age-related hip fractures. Bone 18, S77–S86 (1996).
25. Shumway-Cook, A., Ciol, M. A., Gruber, W. & Robinson, C. Incidence of and risk factors for falls following hip fracture in community-dwelling older adults. Phys. Ther. 85, 648–55 (2005).
26. Curtis, E., Litwic, A., Cooper, C. & Dennison, E. Determinants of Muscle and Bone Aging. J. Cell. Physiol. 230, 2618–2625 (2015).
27. Hassan, E. B. & Duque, G. Osteosarcopenia: A new geriatric syndrome. Aust. Fam. Physician 46, 849–853 (2017).
28. Reginster, J.-Y., Beaudart, C., Buckinx, F. & Bruyère, O. Osteoporosis and sarcopenia. Curr. Opin. Clin. Nutr. Metab. Care 19, 31–36 (2016).
29. Felsenthal, N. & Zelzer, E. Mechanical regulation of musculoskeletal system development. Development 144, 4271–4283 (2017).
30. Hamrick, M. W. A role for myokines in muscle-bone interactions. Exerc. Sport Sci. Rev. 39, 43–7 (2011).
31. Undercarboxylated osteocalcin, muscle strength and indices of bone health in older women. Bone 64, 8–12 (2014).
32. Kim, J. A. et al. Association of serum sclerostin levels with low skeletal muscle mass: The Korean Sarcopenic Obesity Study (KSOS). Bone 128, 115053 (2019).
33. Trajanoska, K. & Rivadeneira, F. The genetic architecture of osteoporosis and fracture risk. Bone 126, 2–10 (2019).
34. Trajanoska, K., Rivadeneira, F., Kiel, D. P. & Karasik, D. Genetics of Bone and Muscle Interactions in Humans. Curr. Osteoporos. Rep. 17, 86–95 (2019).
35. Michou, L. Epigenetics of bone diseases. Jt. Bone Spine 85, 701–707 (2018).
36. Moresi, V., Marroncelli, N., Pigna, E. & Adamo, S. Epigenetics of Muscle Disorders. Med. Epigenetics 315–333 (2016). doi:10.1016/B978-0-12-803239-8.00018-1
37. Medina-Gomez, C. et al. Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/TOM1L2 locus. Nat. Commun. 8, 121 (2017).
38. Guo, Y.-F. et al. Suggestion of GLYAT gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. Hum. Genet. 132, 189–199 (2013).

39. Huang, J. et al. METTL21C Is a Potential Pleiotropic Gene for Osteoporosis and Sarcopenia Acting Through the Modulation of the NF-κB Signaling Pathway. J. Bone Miner. Res. 29, 1531–1540 (2014).
40. Ikram, M. A. et al. The Rotterdam Study: 2018 update on objectives, design and main results. Eur. J. Epidemiol. 32, 807–850 (2017).
41. Rivadeneira, F. et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat. Genet. 41, 1199–1206 (2009).
42. Sudlow, C. et al. UK Biobank: An Open Access Resource for Identifying the Causes of a Wide Range of Complex Diseases of Middle and Old Age. PLOS Med. 12, e1001779 (2015).

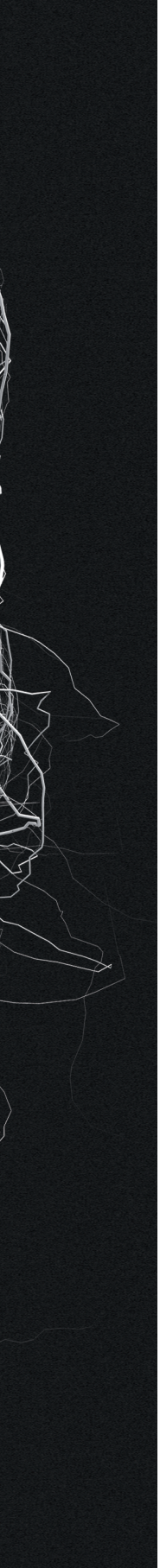




2

Determinants of Osteoporosis and Fracture risk

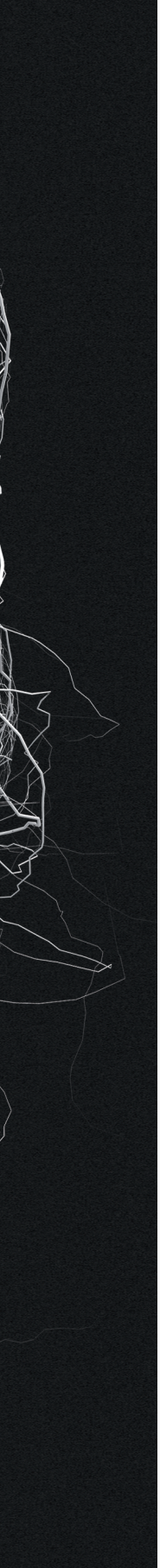




2.1

Epidemiological studies





2.1.1

Fracture incidence and secular trends between 1989 and 2013 in a population-based cohort: The Rotterdam Study

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Bone. *2018 Sep; 114:116-124.*

ABSTRACT

Fracture incidence needs to be evaluated over time to assess the impact of the enlarging population burden of fractures (due to increase in lifespan) and the efficacy of fracture prevention strategies. Therefore, we aimed to evaluate the association of femoral neck bone mineral density (FN-BMD) measured using dual-energy X-ray absorptiometry (DXA) at baseline with fracture risk over a long follow-up time period. Incident non-vertebral fractures were assessed in 14,613 individuals participating in the Rotterdam Study with up to 20 years of follow-up. During a mean follow-up of 10.7 ± 6.2 years, 2971 (20.3%) participants had at least one incident non-vertebral fracture. The risk for any non-vertebral fracture was 1.37 (95% Confidence Interval (CI): 1.25-1.49) and 1.42 (95%CI: 1.35-1.50) for men and women, respectively. The majority (89% in men and 79% in women) of all fractures occurred among participants with a normal or osteopenic T-score. The incidence rates per 1000 person-years for the most common fractures were 5.3 (95%CI: 5.0-5.7) for hip, 4.9 (95%CI: 4.6-5.3) for wrist and 2.3 (95%CI: 2.0-2.5) for humerus. To examine the predictive ability of BMD through follow-up time we determined fracture hazard ratios (HR) per standard deviation decrease in femoral neck BMD across five-year bins. No differences were observed, with a HR of 2.5 (95%CI: 2.0-3.1) after the first 5 years, and of 1.9 (95%CI: 1.1-3.3) after 20 years. To assess secular trends in fracture incidence at all skeletal sites we compared participants at an age of 70–80 years across two time periods: 1989–2001 (n = 2481, 60% women) and 2001–2013 (n = 2936, 58% women) and found no statistically significant difference ($p < 0.05$) between fracture incidence rates (i.e., incidence of non-vertebral fractures of 26.4 per 1000 PY (95%CI: 24.4-28.5)) between 1989 and 2001, and of 25.4 per 1000 PY (95%CI: 23.0-28.0) between 2001 and 2013. In conclusion, BMD is still predictive of future fracture over a long period of time. While no secular changes in fractures rates seem to be observed after a decade, the majority of fractures still occur above the osteoporosis threshold, emphasizing the need to improve the screening of osteopenic patients.

INTRODUCTION

Fractures of the hip, wrist and pelvis are among the most common osteoporotic fractures [1] affecting hundreds of millions of people worldwide. In the European Union, the annual costs of all osteoporotic fractures have been estimated at €37 billion[2] in 2010, of which 54% of the costs are attributed to hip fractures.[2] Due to the aging of industrialized societies, the incidence of osteoporosis and fragility fractures is expected to increase in the years to come.[3, 4]

However, not all studies show a clear increase of fractures rates. For instance, previous studies have yielded conflicting perspectives indicating that the incidence of hip fracture has either increased, plateaued, or even decreased in the last decades.[5-10] Such discrepancies may be explained by multiple factors, including: secular periods in which the fractures occur, changes in clinical practice and drug prescription or compliance, distribution of age and demographics within age and sex strata, migrations and/or geographical origin of the report.[7] For example, studies performed in the USA have reported a decline in the incidence of hip fractures between 1980 and 2000.[8,9] In contrast, in the Netherlands, an initial linear increase in hip fracture incidence during the period between 1972 and 1987[10] is now proposed to have plateaued during the following decades.[11] Moreover, recent information on incidence rates and trends for other sites of non-vertebral fractures is currently lacking despite that non-hip fractures are also associated with higher disability[12] and mortality[13] rates. Depiction of robustly-assessed overall and site-specific fracture trends is important to estimate the burden of osteoporosis and to establish proper and cost-effective prevention strategies.

Part of delineating cost-effective prevention strategies involves determining the predictive ability of fracture risk factors in time. Although fractures have a multifactorial background, apart from age, low BMI and falls, a low bone mineral density (BMD) remains one of the most important risk factors. Studies have shown that the reduction of femoral neck (FN-) BMD is essentially linear over time, comprising ~1-2% of baseline BMD per year.[14] It is well established that BMD can predict fracture risk over a period of 5–10 years[15,16] but little is known[17,18] about the predictive value of BMD over longer periods.

Altogether, our aims were to 1) evaluate the association between BMD at baseline (both continuously and using clinical cut-offs) and fracture risk over a long time period, 2) estimate the incidence of non-vertebral fractures (overall and site-specific) during 23 years of follow-up and 3) evaluate whether incidence rates have changed during two subsequent secular follow-up periods. All analyses were performed for the total group and stratified by sex, considering the well-established sexual differences in fracture rate.

MATERIALS AND METHODS

Study Population

Our study included participants from the Rotterdam Study, an ongoing population-based prospective cohort comprising 14,926 Dutch individuals aged 45 years and older examined across three population sets.[19] At its start in 1990, a total of 7983 participants aged 55 years and older were included in the initial study wave (RSI). The cohort was expanded in 2000 with 3011 participants (RSII) aged 55 years and older; and in 2006 with 3932 participants (RSIII) aged 45 years and older, or who had moved into the study area. All participants underwent an extensive home interview followed by a visit to the research center where various physical and laboratory examinations took place. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare, and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians. From the 14,926 participants, 14,613 provided informed consent to undergo follow-up assessments and were included in the fracture analyses. For the BMD analysis, 3562 participants did not undergo DXA, leaving 11,051 participants for the BMD analyses.

Bone Mineral Density Measurements

FN-BMD (g/cm^2) was measured in RSI-1 (1989-1993) and RSII-1 (2000-2001) using dual energy X-ray absorptiometry (DXA) acquired with a Lunar DPX-L densitometer (Madison, WI, USA). In RSIII-1 (2006-2008), FN-BMD was measured using a GE-Lunar Prodigy bone densitometer. All scans and analyses were performed and verified by a trained technician who applied adjustments when necessary. Sex-specific T-scores (FN-BMD when compared to the young normal reference mean) were calculated using the NHANES III reference population. [20] Osteoporosis was defined according to WHO criteria[21] as a T-score below or equal to -2.5 and osteopenia as a T-score between -1.0 and -2.5. To be able to compare BMD levels with previous reports[15] differences in mean BMD across software versions in RSI-1 (DPX-L and DPX-IQ) for the calculation of T-scores were taken into account to create a uniform dataset of individuals. BMD obtained from the DPX-IQ software was recalculated using the following conversion formulas: BMD = 0.03402 + 0.9371 $*$ DPX-IQ BMD in women and BMD = 0.01588 + 0.9471 $*$ DPX-IQ BMD in men. The intercept and slope values were obtained from regressing BMD DPX-IQ values on the BMD DPX-L values across the same individuals (very similar estimates were obtained when employing different sampling sets of size 500, 1000, 2000 or 5000 individuals to determine the estimates of the calibration).

Incident Fracture Collection

The present analyses are based on follow-up data collected from baseline (1990-1993 for the first cohort, 2000–2001 for the second cohort and 2006-2008 for the third cohort) until December 31, 2012. Follow-up time was calculated as time from baseline to first fracture, death or end of follow-up period (or loss to follow-up), whichever occurred first. All fracture events were reported either by general practitioners (GPs) in the research area by means of computerized systems or through hospital records. Research physicians or trained nurses, who checked patient records regularly, retrieved information from GPs outside the research area. All events reported were verified by research physicians who independently reviewed and coded the information. Subsequently, a medical expert reviewed all inconsistencies in coded events for final classification. For the current study only non-vertebral fractures were considered, as time of event cannot accurately be assed for the vast majority of vertebral fractures.

STATISTICAL ANALYSES

All analyses were performed for the total group of participants and stratified by sex, considering the well-established sex differences in fracture risk.[22-24] First, Cox proportional hazard models adjusted for age at baseline and cohort were used to estimate the hazard ratio (HR) of first fracture associated with 1 SD decrease in FN-BMD across 1) all non-vertebral fractures, 2) for specific types of fractures and 3) all non-vertebral fractures in groups of subjects classified by WHO-based BMD cut-offs. The latter analyses were done in three age groups: below 65 years; 65-75 years; and 75 years and older. Subjects classified as having BMD in the normal range (T-score > 1.0) were used as the reference group. In order to better examine the fracture prediction we performed sensitivity analyses in which the follow-up time was stratified by 5-years categories: 0-5; 5-10; 10-15; 15-20; and > 20 years. The proportionality of hazards assumption of Cox-models was checked using correlation tests of Schoenfeld residuals. For the non-vertebral fractures analysis, we used natural splines to fit a proportional hazards model in order to smooth the nonlinear effect of age. Second, fracture incidence rates were calculated according to fracture site and additionally subdivided into three main categories: upper extremity; lower extremity; and other fractures. Incidence rates were expressed as number of fractures per 1000 person-years (PY) with 95% confidence intervals, estimated using the exact Poisson formula.[25] Third, to draw the comparison between secular periods (1989-2001 [N = 2481] and 2001-2013 [N = 2937]) incidence rates were calculated for men and women who were 70-80 years old at the start of the respective follow-up period. We used a distinct age category in order to avoid bias due to different age distribution among the follow-ups. The

70-80 years of age category was used, as it comprises the period in life with greatest exponential increase in fracture rates of most likely osteoporotic etiology. Poisson exact test was used to test the null hypothesis that the ratio between the rates was equal to 1. The trend p-value was adjusted for multiple testing. All analyses were performed using the statistical package **R version 3.1.2**.

RESULTS

Characteristics of the Study Population

Figure 1 is a flow diagram describing the selection of study participants. At baseline, the mean (± SD) age of the participants was 64.7 (±9.4) years for men and 66.5 (±10.9)

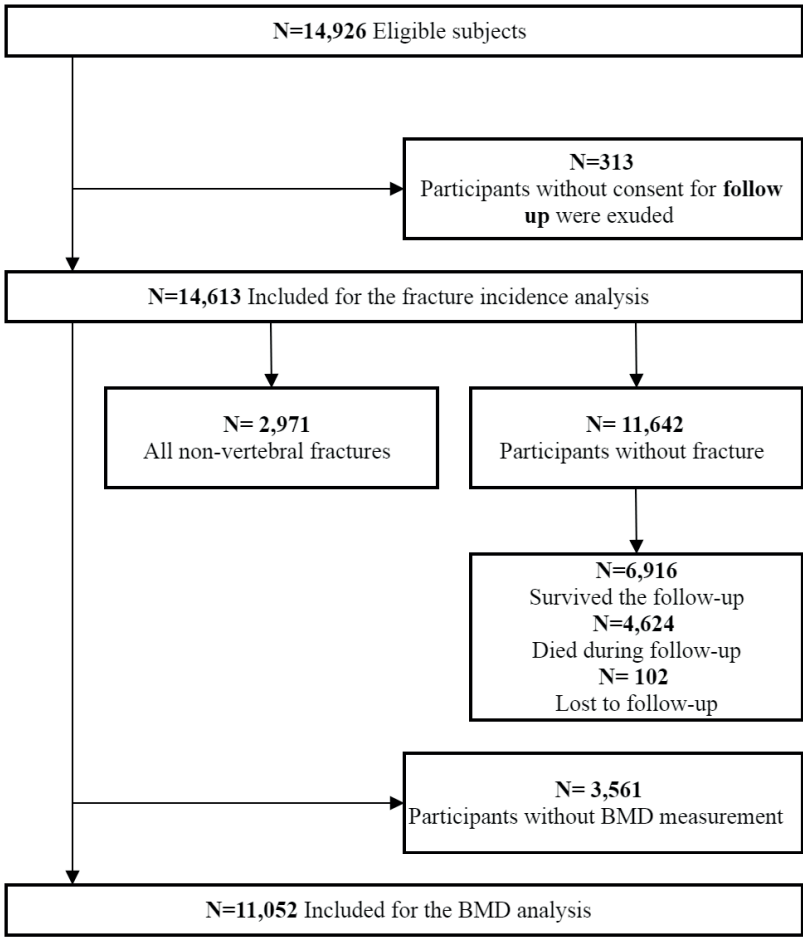


Figure 1 | Flow chart of participants in the study

years for women. The prevalence of osteoporosis (BMD T-score < -2.5) was 10.8% in women and 6.6% in men. The prevalence increased exponentially with age for all types of fractures but this relation is less prominent for wrist fractures (**Figure 2**). Above the age of 85 years, 40.9% of the women and 31.7% of the men had osteoporosis. Nearly 21% of all non-vertebral fractures occurred in women with osteoporosis and 12% in osteoporotic men (**Figure 3** and **Supplementary Table 3**). In women and men with osteoporosis, hip fractures were the most common type of fractures (38% women and 29% men). Overall, the highest fraction of all and -site-specific fractures (above 50%) occurred in men and women with osteopenia.

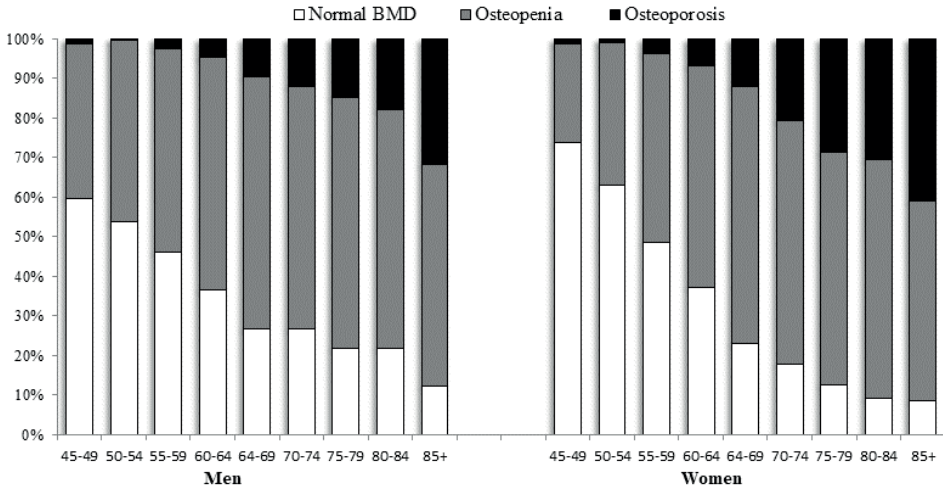


Figure 2 | Prevalence of osteoporosis and osteopenia in men and women by gender-specific T-scores. Hip fractures occurred more often in men and women above the age of 75 years. Overall, across the age distribution hip fracture incidence rates in men were similar to the rates of women 5 year younger. Furthermore, women had higher incidence rates than men in the same age category for any type of fracture.

Association between BMD and Incident Fracture

Associations of FN-BMD with non-vertebral fractures are shown in Table 1. For every SD decrease in BMD a 1.46 [95%CI: 1.34-1.46] times increased risk in non-vertebral fracture risk was observed. The associations of BMD with fracture risk were similar in women (1.42 [95%CI: 1.35-1.50]) and men (1.37 [95%CI: 1.25-1.49]). Low BMD (T-score < 1) was strongly associated with increases site-specific fracture risk at the hip, wrist and proximal humerus, with similar effects seen in women and men (**Table 1**). Individuals with osteopenia or osteoporosis had 1.4 to 4 times higher risk of non-vertebral fracture than individuals with normal BMD (**Figure 4**). Women above the age of 75 with osteoporosis had 3.8 times increased risk to develop fractures, while the risk in older women with osteopenia was 1.8 times higher than older women without

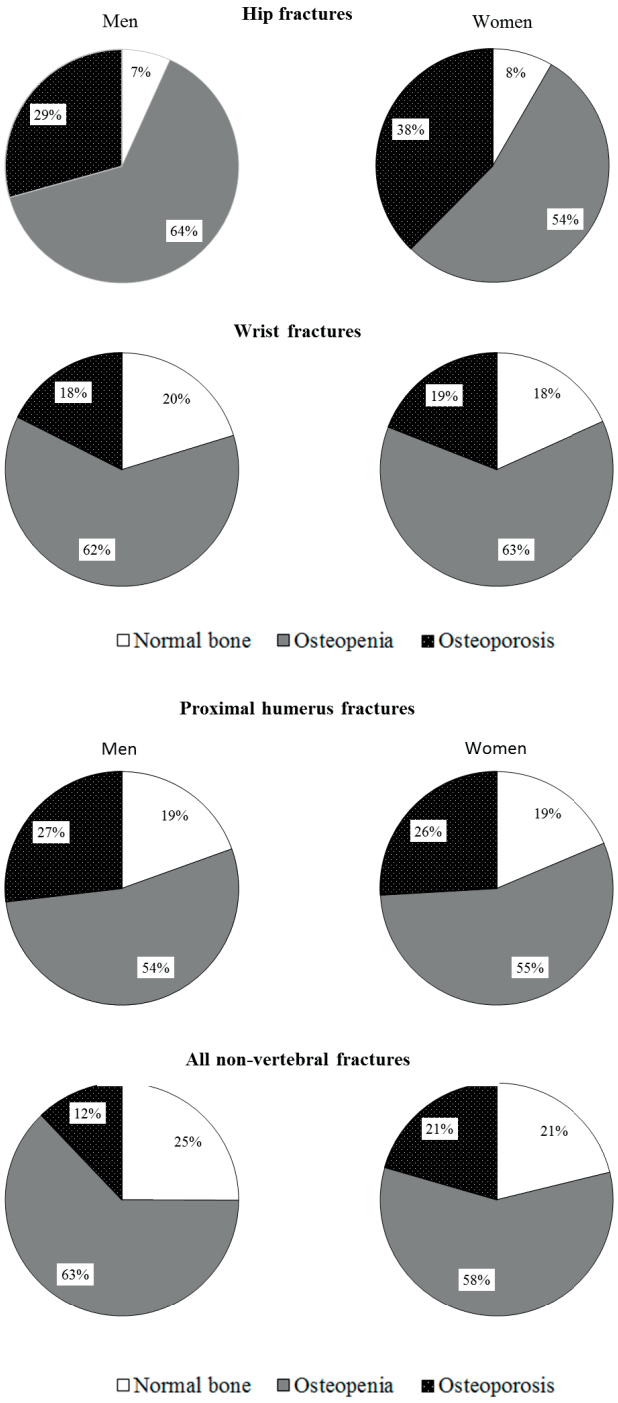


Figure 3 | Percentages of non-vertebral, hip, proximal humerus and wrist fractures that occurred in men and women with osteoporosis, osteopenia or normal BMD using gender-specific T-scores.

Table 1 | Age-adjusted hazard ratio (95%CI) for the predictive value of femoral neck BMD (g/cm^2) per one SD decrease.

Type of fracture	Total (n=11,052)			Men (n=4,777)			Women (n=6,275)		
	Number	Hazard ratio	95%CI	Number	Hazard Ratio	95%CI	Number	Hazard ratio	95%CI
Upper extremities									
Proximal humerus	261	1.59	1.38-1.83	41	1.85	1.31-2.61	220	1.55	1.33-1.81
Wrist	578	1.45	1.32-1.59	74	1.73	1.34-2.23	504	1.42	1.29-1.57
Hand	262	1.30	1.14-1.49	92	1.3	1.04-1.63	170	1.29	1.09-1.53
Other proximal arm [a]	140	1.48	1.23-1.79	43	1.3	0.94-1.79	97	1.59	1.26-2.00
Other distal arm [b]	121	1.59	1.07-1.59	20	1.36	0.84-2.23	101	1.3	1.04-1.62
Lower extremities									
Pelvis	139	1.52	1.261.84	33	1.23	0.85-1.78	106	1.63	1.30-2.04
Hip	564	2.05	1.86-2.26	133	2.3	1.89-2.82	431	1.97	1.76-2.21
Ankle	106	0.99	0.81-1.21	27	1.09	0.73-1.62	79	0.96	0.76-1.22
Metatarsal	123	1.28	1.05-1.55	21	1.82	1.12-2.94	102	1.22	0.99-1.51
Other foot [c]	165	1.14	0.97-1.35	36	1.26	0.89-1.80	129	1.12	0.93-1.35
Other proximal leg [d]	41	1.98	1.37-2.87	8	1.38	0.64-2.94	33	2.2	1.44-3.37
Other distal leg [e]	166	1.14	0.97-1.34	46	1.03	0.76-1.41	120	1.19	0.98-1.45
Other[f]	263	1.20	1.05-1.37	122	1.07	0.89-1.29	141	1.31	1.09-1.58
All non-vertebral	2,233	1.40	1.34-1.46	586	1.37	1.25-1.49	1647	1.42	1.35-1.50

[a] other proximal arm fractures include fractures of the scapula, clavicula and non-proximal fractures of the humerus

[b] other distal arm fractures of the other parts of the radius and/or ulna

[c] other foot fractures include non-metatarsal foot fractures

[d] other proximal leg fractures include non-hip fractures of the femur and patella

[e] other distal leg fractures include non-ankle fractures of the tibia and/ or fibula

[f] other fractures include skull, rib and sternum

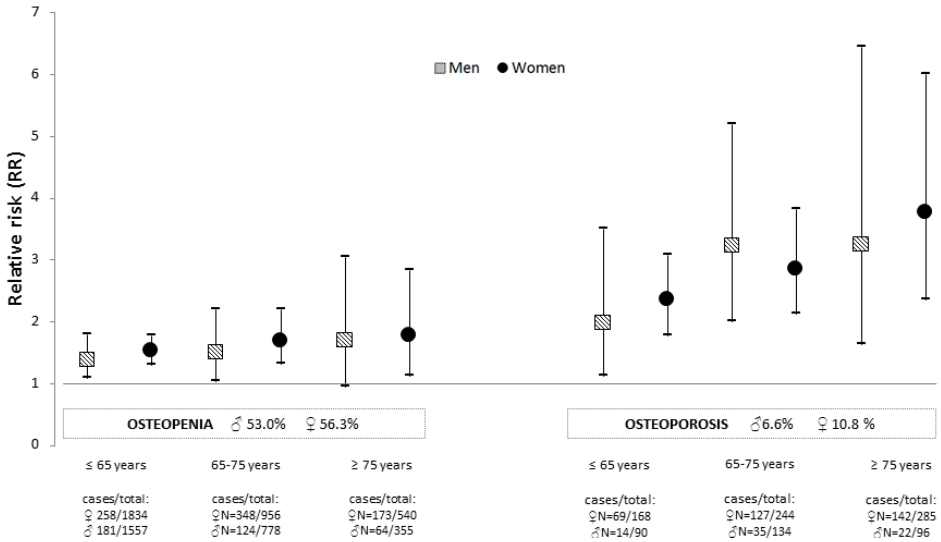


Figure 4 | Relative fracture risk comparing subjects with osteopenia or osteoporosis with normal bone subjects stratified by sex and age.

osteopenia/osteoporosis. The analysis of fracture risk over different follow-up periods confirms that BMD remains a good predictor of hip and non-vertebral fractures in time. In the first 5 years the fracture HR per SD decrease in BMD was 2.5 (95%CI: 2.0-3.1), while after 20 years the HR was 1.9 (95%CI: 1.1-3.3) (**Supplementary Table 1**).

Fracture Incidence Rates

During the 23 years of follow-up (overall mean: 10.7 ± 6.2; RSI-1 = 14.5, RSII-1 = 12.0, RSIII-1 = 5.6 years), 2971 (20.3%) participants sustained at least one non-vertebral fracture (of whom 75.4% were women). **Table 2** shows the fracture incidence rates by site and sex. Overall, the incidence of non-vertebral fractures was 12.4 [95%CI:

Table 2 | Gender and site-specific incidence rates of first non-vertebral fractures per 1000 person years.

Type of fracture	Total (n=14,613)			Men (n=6,024)			Women (n=8,589)		
	Number	Incidence rate	95%CI	Number	Incidence rate	95%CI	Number	Incidence rate	95%CI
Upper extremities									
Proximal humerus	356	2.29	2.07-2.54	59	0.96	0.74-1.23	297	3.18	2.83-3.56
Wrist	753	4.96	4.61-5.32	93	1.51	1.23-1.85	660	7.30	6.76-7.88
Hand	316	2.04	1.83-2.28	106	1.73	1.43-2.09	210	2.24	1.96-2.57
Other proximal arm [a]	195	1.25	1.08-1.44	55	0.89	0.68-1.16	140	1.48	1.26-1.75
Other distal arm [b]	155	0.99	0.85-1.16	25	0.40	0.27-0.60	130	1.38	1.16-1.64
Lower extremities									
Pelvis	194	1.24	1.08-1.43	42	0.68	0.50-0.92	152	1.61	1.37-1.88
Hip	830	5.37	5.02-5.75	185	3.01	2.60-3.47	645	6.94	6.43-7.50
Ankle	131	0.84	0.71-0.99	30	0.48	0.34-0.69	101	1.07	0.88-1.30
Metatarsal	149	0.95	0.81-1.12	24	0.39	0.26-0.58	125	1.33	1.11-1.58
Other foot [c]	194	1.24	1.08-1.43	44	0.71	0.53-0.96	150	1.59	1.36-1.87
Other proximal leg [d]	70	0.46	0.35-0.56	11	0.18	0.10-0.32	59	0.62	0.48-0.80
Other distal leg [e]	219	1.41	1.23-1.60	50	0.81	0.61-1.07	169	1.80	1.54-2.09
Other[f]	329	2.12	1.90-2.36	139	2.27	1.92-2.67	190	2.02	1.75-2.33
All non-vertebral	2,971	21.35	20.60-22.14	731	12.54	11.66-13.48	2240	27.72	26.59-28.89

[a] other proximal arm fractures include fractures of the scapula, clavicula and non-proximal fractures of the humerus

[b] other distal arm fractures of the other parts of the radius and/or ulna

[c] other foot fractures include non-metatarsal foot fractures

[d] other proximal leg fractures include non-hip fractures of the femur and patella

[e] other distal leg fractures include non-ankle fractures of the tibia and/ or fibula

[f] other fractures include skull, rib and sternum

11.6-13.4] per 1000 PY in men and 27.3 [95%CI: 26.2-28.4] per 1000 PY in women. In both men and women, the most predominant fracture sites were the wrist, hand, proximal humerus and hip. Apart from the fracture rates of the proximal humerus in men, all other non- vertebral and site-specific fracture rates increased exponentially with age (**Figure 5**).

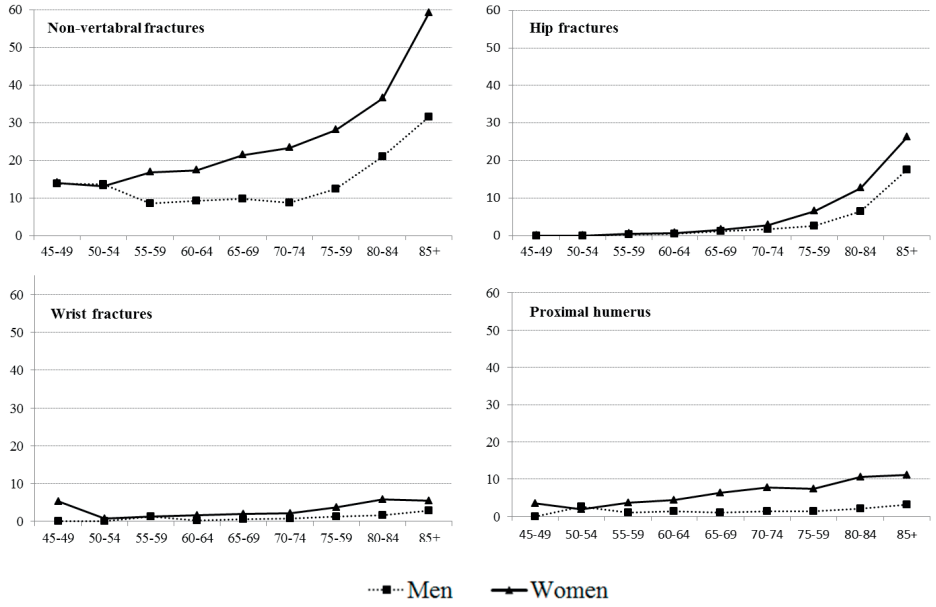


Figure 5 | Age related incidence of all non-vertebral, hip, wrist and proximal humerus fractures in men and women.

Comparing Fracture Rate between 1989 and 2001 and 2001–2013

The overall incidence of non-vertebral fractures in the period between 1989 and 2001 (25.4 per 1000 PY [95%CI: 23.0–28.0]) was similar to those observed in the period between 2001 and 2013 (26.4 per 1000 PY [95%CI: 24.4-28.5]) (**Supplementary Table 3A and B**). There was no significant change in site-specific fracture trends, with the exception of an 8.3% increased distal leg fracture incidence (p = 0.02) in men (**Figure 6**). However, this trend is not significant (0.05/14, P= 0.004) after taking multiple testing into account.

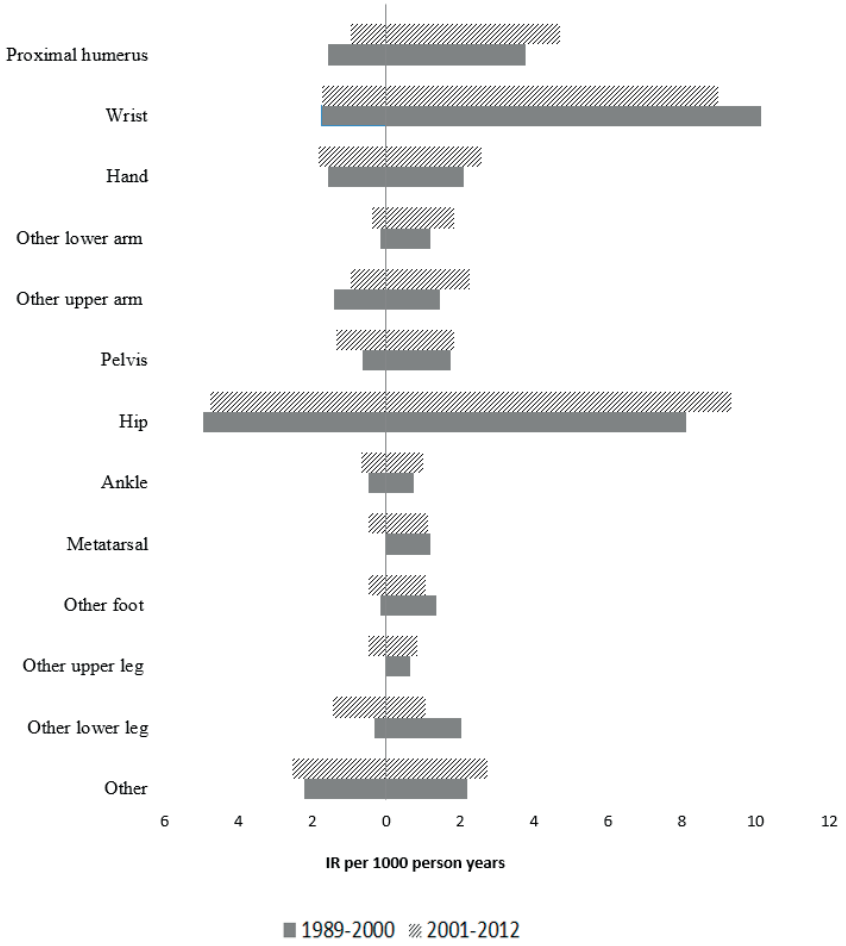


Figure 6 | Incidence of site-specific fractures in 1989 to 2001 compared with 2001 to 2013 (70–80 years old) stratified by sex

DISCUSSION

In this population-based prospective cohort, a single FN-BMD measurement at baseline remains a strong predictor of incident non-vertebral fragility fractures over a period of 20 years. The overall incidence rate of suffering non-vertebral fractures was 21.1 per 1000 PY [95%CI: 20.3-21.9] with a higher incidence rate in women than in men. The most frequent non-vertebral fractures in elderly men and women continues to be fractures of the hip, wrist, proximal humerus and hand, with most events occurring above the osteoporosis diagnostic and intervention threshold (T-score above

-2.5). We observed no secular trend changes in fracture incidence between 1989 and 2001 and 2001-2013.

In an earlier effort in the Rotterdam Study,[15] we showed an in-verse association between FN-BMD and the risk of all non-vertebral fractures during a mean 6.8-year follow-up period. After extending this follow-up period in the current study by 10 more years on average, we conclude that using one single BMD measurement is still a good predictor for fracture risk even up to 20 years after. This finding reinforces the central role of BMD in fracture etiology and prediction. Our findings corroborate with findings recently reported by Black and colleagues[17] among women of the Study of Osteoporotic Fractures showing that a single FN-BMD measurement strongly predicts long-term hip fracture up to 23 years. The fracture risk in the first five years (0–5 y) was comparable to the risk after 20 years (20-23 y). Additionally, we showed that low BMD is not only associated with hip fractures but also with fractures at other skeletal sites, among other peripheral sites. However, BMD did not have any predictive value for ankle and foot fractures. Furthermore, individuals with osteoporosis (BMD T-score < -2.5) fractured two to three times more often than individuals with normal BMD from the same sex and age group. In addition, in our study women with osteoporosis fractured more often than men, while the proportion of fractures in the osteopenia group is similar in men and women. Although BMD is an important predictor of fracture risk, the treatment indication based on T-score cut-offs alone it is not sufficient. As in our previous work, we still see that if we use a T-score of −2.5 as a diagnostic and treatment threshold, a very large proportion (> 50%) of the individuals who fracture will remain untreated; fuelling additionally the existing crisis in osteoporosis treatment.[26,27] Although there is a consensus to treat osteopenic patients presenting with prevalent low-energy fractures, the treatment of osteopenia without a fracture remains controversial. Risk assessments tools like FRAX[28] and GARVAN[29] have been developed in the last decade, which take into account other time-dependent clinical risk factors which may establish indication for pharmacotherapy. For example, age is an important risk factor and studies have found that drug-based interventions among elderly (≥65 years) women with osteopenia are cost-effective.[30] However, treatment of elderly women with osteopenia is still not part of current clinical practice recommendations and most guidelines for osteopenic patients emphasize lifestyle changes. Nevertheless, our findings encountering no significant change in the secular trends of fracture risk across periods, suggest that (at least in the Netherlands) additional steps are required to improve fracture risk prediction. Redefining intervention thresholds and targeting strategies to improve adherence to guidelines and drug compliance are some of the ways to achieve this improvement.

As reported in previous studies[10,31,32] we observed that wrist and hip fractures occur more frequently in women than in men, with a ratio of 2:1 and 5:1 for hip and

wrist fractures, respectively. The sex-specific incidence of hip fractures was similar to a previous report in the Dutch population[10] performed in 1987. Using the Dutch medical register they observed that for every 100,000 inhabitants 669 women and 308 men suffered a hip fracture. The observed sexual dimorphism in fracture risk has been mainly attributed to the rapid postmenopausal bone loss in women, accounting for 20–30% loss in cancellous bone and 5–10% loss in cortical bone.[33] Additionally, higher peak bone mass and stronger bone geometry parameters in men contribute to the sex differences.

Beside the differences in sex, large variation in hip fracture rates (up to 7-fold) has been observed across countries. Ismail and colleagues[34] estimated site-specific fracture incidence rates across Europe and found differences in rates by sex and region. In line with our study they have reported similar sex-specific hip fracture rates in Scandinavia and lower in Southern, Eastern and Western Europe. Moreover, in women the incidence rate of humerus fractures followed the same country pattern as hip fracture, while in men the incidence rate of our study was only higher than in Eastern Europe and similar to the other countries. No differences in wrist fracture incidence rates were noticed between regions. Beside falls and higher latitude, other environmental factors together with genetic factors could lead to geographical variations in incidence rates. Still, direct comparison of rates across studies should be made with caution given differences in age and sex distribution, methods for age standardization, fracture ascertainment and regional variation in general health.

In our study we provided incidence rates for different site-specific fractures. Although the major osteoporotic fractures (hip, spine and forearm) are considered the most important and prominent ones, other site-specific fractures also contribute considerably to the burden of osteoporosis. These other fractures account for 31.5% of all health care expenditure,[35] while it has also been shown that women with other types of fracture (non-hip) utilize more healthcare resources compared to women with hip fracture.[36] Altogether, gaining additional insight and knowledge about the incidence of site-specific fractures and trends in time might help in establishing better prevention strategies.

Studies in different countries have shown a steady increase in the incidence of hip fractures in the 80s and early 90s. [37-39] But recently, it has been proposed that the incidence of fracture has remained rather stable or could have even decreased in some countries (USA, Canada, Australia and Switzerland).[40-42] In our study, although there was a positive non-significant fracture trend for the majority of site-specific fractures, the incidence rates have remained rather stable over the past 20 years. Amin and colleagues[43] have also reported stabilization of the fracture incidence rates at many skeletal sites in the past two decades in the U.S. This is in contrast with findings from a recent national registry-based study in the Netherlands which observed 3-4%

increased incidence rates for wrist, hand/finger, hip/proximal leg, ankle and foot/toe fractures.[44] Moreover, Nanniga and colleagues[45] have reported increased incidence rate of pelvic fractures in the Netherlands. The different findings from the latter two studies may be attributed to a couple of factors. Firstly, the studies used hospital records to extrapolated data while we used both hospital and GP records. Next, in the study by Beerekamp et al.[44] the age range was wide and included patients ≥16 years old. In addition, their population mean age increased from 2004 to 2014 which could be one reason for the increase in the absolute number of fractures. Reason why in our study we chose a fixed age category of 70-80 years to compare between two time periods. Further, they had missing data on 7% in elderly above 70 years which could have influenced the fracture numbers. Although all events were ICD10 or ICD9 codes different group-classifications applied between the studies might have impacted the rates. Finally, our study is a cohort study and the findings might be specific for our population only. Overall, variation in fracture rates in time can be due to birth cohort effects resulting from changes in early life risk factors such as perinatal nutrition, behavioral changes in smoking and alcohol consumption, BMI, treatment of osteoporosis [bisphosphonates, calcium, vitamin D] or use of estrogen therapy. Analysis of the trends can help us identify the main factors leading to differences between countries.

Our study has several strengths. We were able to estimate the incidence and incidence trends of site-specific fractures in both men and women from the same population after a prolonged follow-up. In addition, in the Netherlands, the only way to access specialist and hospital care is consulting a GP. Therefore, checking the GPs' medical records of all participants should have resulted in a more comprehensive follow-up. One important limitation is that our study may be subject to selection bias at baseline due to non-response of subjects with impaired mobility and this could have led to an underestimation of the incidence rates. Loss to follow-up and mortality can also cause lower incidence rates, given that usually these individuals are older and more susceptible to bone loss. Competing risk of death, changes in clinical practice and bisphosphonates use could have influenced the fracture rates in both follow-up periods. However, we are not able to account for these changes in our study. Moreover, we were not able to use repeated BMD measures due to differences in densitometer per follow-up. Although single BMD measurement is associated with fracture risk over longer period in the population, repeated BMD measures may still be necessary to improve the prediction of fracture for an individual.[46]

In conclusion, BMD remains a strong predictor of hip and non-vertebral fractures over 20 years in both men and women. The majority of fractures continue to occur above the osteoporosis threshold emphasizing the need to improve the screening of osteopenic patients. Most importantly, we established there are no difference in fracture trends between the periods of 1989-2001 and 2001-2013, welcoming active

actions seeking to improve the diagnoses, treatment and prevention strategies to this costly disease.

Detailed acknowledgments and online resources can be found in the published article online

https://www.sciencedirect.com/science/article/pii/S8756328218302333?via%3Dihub

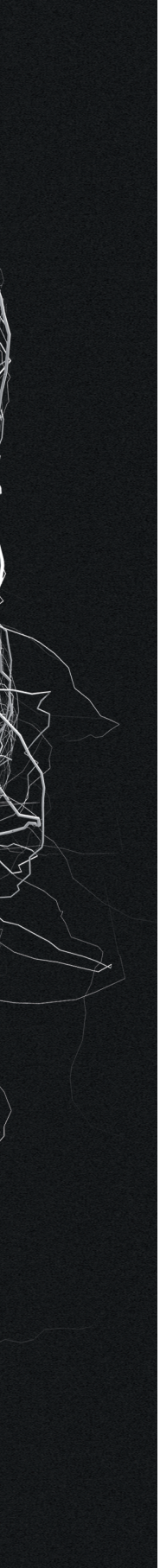
REFERENCES

1. A. Litwic, M. Edwards, C. Cooper, E. Dennison, Geographic differences in fractures among women, Women's Health (Lond. Engl.) 8 (2012) 673–684, http://dx.doi. org/10.2217/whe.12.54.
2. E. Hernlund, A. Svedbom, M. Ivergård, J. Compston, C. Cooper, J. Stenmark, et al., Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA), Arch. Osteoporos. 8 (2013) 136, http://dx.doi.org/ 10.1007/s11657-013-0136-1.
3. B. Gullberg, O. Johnell, J.A. Kanis, World-wide projections for hip fracture, Osteoporos. Int. 7 (1997) 407–413.
4. R. Burge, B. Dawson-Hughes, D.H. Solomon, J.B. Wong, A. King, A. Tosteson, Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025, J. Bone Miner. Res. 22 (2007) 465–475, http://dx.doi.org/10. 1359/jbmr.061113.
5. M.-J. Pueyo-Sánchez, M. Larrosa, X. Suris, E. Casado, J. Auleda, J. Fusté, et al., Secular trend in the incidence of hip fracture in Catalonia, Spain, 2003–2014, Age Ageing 46 (2016) 324–328, http://dx.doi.org/10.1093/ageing/afw196.
6. A.J. Søgaard, K. Holvik, H.E. Meyer, G.S. Tell, C.G. Gjesdal, N. Emaus, et al., Continued decline in hip fracture incidence in Norway: a NOREPOS study, Osteoporos. Int. 27 (2016) 2217–2222, http://dx.doi.org/10.1007/s00198-016-3516-8.
7. S.R. Cummings, L.J. Melton, Epidemiology and outcomes of osteoporotic fractures, Lancet 359 (2002) 1761–1767, http://dx.doi.org/10.1016/S0140-6736(02)08657-9.
8. R. Hiebert, G.B. Aharonoff, E.L. Capla, K.A. Egol, J.D. Zuckerman, K.J. Koval, Temporal and geographic variation in hip fracture rates for people aged 65 or older, New York State, 1985–1996, Am. J. Orthop. (Belle Mead NJ) 34 (2005) 252–255.
9. D. Zingmond, L.J. Melton, S. Silverman, Increasing hip fracture incidence in California Hispanics, 1983 to 2000, Osteoporos. Int. 15 (2004) 603–610, http://dx. doi.org/10.1007/s00198-004-1592-7.
10. F.T. Boereboom, R.R. de Groot, J.A. Raymakers, S.A. Duursma, The incidence of hip fractures in The Netherlands, Neth. J. Med. 38 (1991) 51–58.
11. W.G. Goettsch, R.B. de Jong, P. Kramarz, R.M.C. Herings, Developments of the incidence of osteoporosis in The Netherlands: a PHARMO study, Pharmacoepidemiol. Drug Saf. 16 (2007) 166–172, http://dx.doi.org/10.1002/pds. 1245.
12. H.A. Fink, K.E. Ensrud, D.B. Nelson, R.P. Kerani (Formerly Pieper), P.J. Schreiner, Y. Zhao, et al., Disability after clinical fracture in postmenopausal women with low bone density: the fracture intervention trial (FIT), Osteoporos. Int. 14 (2003) 69–76, http://dx.doi.org/10.1007/s00198-002-1314-y.
13. T. Tran, D. Bliuc, T. van Geel, J.D. Adachi, C. Berger, J. van den Bergh, et al., Population-wide impact of non-hip non-vertebral fractures on mortality, J. Bone Miner. Res. 32 (2017) 1802–1810, http://dx.doi.org/10.1002/jbmr.3118.
14. L.J. Melton, E.J. Atkinson, M.K. O'Connor, W.M. O'Fallon, B.L. Riggs, Determinants of bone loss from the femoral neck in women of different ages, J. Bone Miner. Res. 15 (2000) 24–31, http://dx.doi.org/10.1359/jbmr.2000.15.1.24.

15. S.C.E. Schuit, M. van der Klift, A.E.A.M. Weel, C.E.D.H. de Laet, H. Burger,E. Seeman, et al., Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study, Bone 34 (2004) 195–202.
16. L.J. Melton, C.S. Crowson, W.M. O'Fallon, H.W. Wahner, B.L. Riggs, Relative con-tributions of bone density, bone turnover, and clinical risk factors to long-term fracture prediction, J. Bone Miner. Res. 18 (2003) 312–318, http://dx.doi.org/10. 1359/jbmr.2003.18.2.312.
17. D.M. Black, J.A. Cauley, R. Wagman, K. Ensrud, H.A. Fink, T.A. Hillier, et al., The ability of a single BMD and fracture history assessment to predict fracture over 25 years in post-menopausal women: the study of osteoporotic fractures, J. Bone Miner. Res. (2017), http://dx.doi.org/10.1002/jbmr.3194.
18. E. Sornay-Rendu, F. Duboeuf, S. Boutroy, R.D. Chapurlat, How to predict fragility fracture beyond 10 years? The OFELY study, J. Clin. Endocrinol. Metab. 99 (2014) 4690–4697, http://dx.doi.org/10.1210/jc.2014-2601.
19. M.A. Ikram, G.G.O. Brusselle, S.D. Murad, C.M. van Duijn, O.H. Franco, A. Goedegebure, et al., The Rotterdam Study: 2018 update on objectives, design and main results, Eur. J. Epidemiol. 32 (2017) 807–850, http://dx.doi.org/10.1007/ s10654-017-0321-4.
20. A.C. Looker, H.W. Wahner, W.L. Dunn, M.S. Calvo, T.B. Harris, S.P. Heyse, et al., Updated data on proximal femur bone mineral levels of US adults, Osteoporos. Int. 8 (1998) 468–489.
21. J.A. Kanis, Assessment of fracture risk and its application to screening for post-menopausal osteoporosis: synopsis of a WHO report. WHO Study Group, Osteoporos. Int. 4 (1994) 368–381.
22. E. Lobo, G. Marcos, J. Santabárbara, H. Salvador-Rosés, L. Lobo-Escolar, C. De la Cámara, et al., Gender differences in the incidence of and risk factors for hip fracture: a 16-year longitudinal study in a southern European population, Maturitas 97 (2017) 38–43, http://dx.doi.org/10.1016/j.maturitas.2016.12.009.
23. N.D. Nguyen, H.G. Ahlborg, J.R. Center, J.A. Eisman, T.V. Nguyen, Residual life-time risk of fractures in women and men, J. Bone Miner. Res. 22 (2007) 781–788, http://dx.doi.org/10.1359/jbmr.070315.
24. P.M. Cawthon, Gender differences in osteoporosis and fractures, Clin. Orthop. Relat. Res. 469 (2011) 1900–1905, http://dx.doi.org/10.1007/s11999-011-1780-7.
25. K. Ulm, A simple method to calculate the confidence interval of a standardized mortality ratio (SMR), Am. J. Epidemiol. 131 (1990) 373–375.
26. N. Binkley, R.D. Blank, W.D. Leslie, E.M. Lewiecki, J.A. Eisman, J.P. Bilezikian, Osteoporosis in crisis: it's time to focus on fracture, J. Bone Miner. Res. 32 (2017) 1391–1394, http://dx.doi.org/10.1002/jbmr.3182.
27. S. Khosla, E. Shane, A crisis in the treatment of osteoporosis, J. Bone Miner. Res. 31 (2016) 1485–1487, http://dx.doi.org/10.1002/jbmr.2888.
28. J.A. Kanis, D. Hans, C. Cooper, S. Baim, J.P. Bilezikian, N. Binkley, et al., Interpretation and use of FRAX in clinical practice, Osteoporos. Int. 22 (2011) 2395–2411, http://dx.doi.org/10.1007/s00198-011-1713-z.
29. N.D. Nguyen, S.A. Frost, J.R. Center, J.A. Eisman, T.V. Nguyen, Development of prognostic nomograms for individualizing 5-year and 10-year fracture risks, Osteoporos. Int. 19 (2008) 1431–1444, http://dx.doi.org/10.1007/s00198-008-0588-0.
30. E.S. Meadows, R. Klein, M.D. Rousculp, L. Smolen, R.L. Ohsfeldt, J.A. Johnston, Cost-effectiveness of preventative therapies for postmenopausal women with osteopenia, BMC Womens Health 7 (2007) 6, , http://dx.doi.org/10.1186/1472-6874-7-6.

31. B.R. Singer, G.J. Mclauchlan, C.M. Robinson, J. Christie, Epidemiology of fractures in 15 000 adults: the influence of age and gender, J. Bone Joint Surg. (Br.) 8080 (1998) 243–248.
32. J.A. Baron, M. Karagas, J. Barrett, W. Kniffin, D. Malenka, M. Mayor, et al., Basic epidemiology of fractures of the upper and lower limb among Americans over 65 years of age, Epidemiology 7 (1996) 612–618.
33. C.J. Rosen, American Society for Bone and Mineral Research. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, American Society for Bone and Mineral Research, 2009.
34. A.A. Ismail, S.R. Pye, W.C. Cockerill, M. Lunt, A.J. Silman, J. Reeve, et al., Incidence of limb fracture across Europe: results from the European Prospective Osteoporosis Study (EPOS), Osteoporos. Int. 13 (2002) 565–571, http://dx.doi.org/10.1007/ s001980200074.
35. P.D. Delmas, F. Marin, R. Marcus, D.A. Misurski, B.H. Mitlak, Beyond hip: im-portance of other nonspinal fractures, Am. J. Med. 120 (2007) 381–387, http://dx. doi.org/10.1016/j. amjmed.2006.06.003.
36. G. Ioannidis, J. Flahive, L. Pickard, A. Papaioannou, R.D. Chapurlat, K.G. Saag, et al., Non-hip, non-spine fractures drive healthcare utilization following a fracture: The Global Longitudinal Study of Osteoporosis in Women (GLOW), Osteoporos. Int. 24 (2013) 59–67, http:// dx.doi.org/10.1007/s00198-012-1968-z.
37. J. Joseph Melton, W.M. O'Fallon, B.L. Riggs, Secular trends in the incidence of hip fractures, Calcif. Tissue Int. 41 (1987) 57–64, http://dx.doi.org/10.1007/ BF02555245.
38. C. Cooper, Z.A. Cole, C.R. Holroyd, S.C. Earl, N.C. Harvey, E.M. Dennison, et al., Secular trends in the incidence of hip and other osteoporotic fractures, Osteoporos. Int. 22 (2011) 1277–1288, http://dx.doi.org/10.1007/s00198-011-1601-6.
39. L.J. Melton, P.C. Amadio, C.S. Crowson, W.M. O'Fallon, Long-term trends in the incidence of distal forearm fractures, Osteoporos. Int. 8 (1998) 341–348, http://dx. doi.org/10.1007/ S001980050073.
40. J.A. Stevens, R. Anne Rudd, Declining hip fracture rates in the United States, Age Ageing 39 (2010) 500–503, http://dx.doi.org/10.1093/ageing/afq044.
41. K.P. Chang, J.R. Center, T.V. Nguyen, J.A. Eisman, Incidence of hip and other os-teoporotic fractures in elderly men and women: Dubbo Osteoporosis Epidemiology Study, J. Bone Miner. Res. 19 (2004) 532–536, http://dx.doi.org/10.1359/JBMR. 040109.
42. W.D. Leslie, M. Sadatsafavi, L.M. Lix, M. Azimaee, S. Morin, C.J. Metge, et al., Secular decreases in fracture rates 1986–2006 for Manitoba, Canada: a population-based analysis, Osteoporos. Int. 22 (2011) 2137–2143, http://dx.doi.org/10.1007/ s00198-010-1470-4.
43. S.C.M. Amin, S.J. Achenbach, E.J. Atkinson, S. Khosla, L.J. Melton Iii, Trends in fracture incidence: a population-based study over 20 years, J. Bone Miner. Res. 29 (2014) 581–589, http://dx.doi.org/10.1002/jbmr.2072.
44. M.S.H. Beerekamp, R.J.O. de Muinck Keizer, N.W.L. Schep, D.T. Ubbink, M.J.M. Panneman, J.C. Goslings, Epidemiology of extremity fractures in the Netherlands, Injury 48 (2017) 1355–1362, http://dx.doi.org/10.1016/j.injury. 2017.04.047.
45. G.L. Nanninga, K. de Leur, M.J.M. Panneman, M. van der Elst, K.A. Hartholt, Increasing rates of pelvic fractures among older adults: the Netherlands,
46. 1986–2011, Age Ageing 43 (2014) 648–653, http://dx.doi.org/10.1093/ageing/ aft212.
47. T.V. Nguyen, J.A. Eisman, Assessment of fracture risk: population association versus individual prediction, J. Bone Miner. Res. (2018), http://dx.doi.org/10.1002/jbmr. 3367.





2.1.2

The Association between Metabolic Syndrome, Bone Mineral Density, Hip Bone Geometry and Fracture Risk: The Rotterdam Study

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ABSTRACT

The association between metabolic syndrome (MS) and bone health remains unclear. We aimed to study the association between MS and hip bone geometry (HBG), femoral neck bone mineral density (FN-BMD), and the risk of osteoporosis and incident fractures. Data of 2040 women and 1510 men participants in the third visit (1997–1999) of the Rotterdam Study (RSI-3), a prospective population-based cohort, were available (mean follow-up 6.7 years). MS was defined according to the recent harmonized definition. HBG parameters were measured at the third-round visit whereas FN-BMD was assessed at the third round and 5 years later. Incident fractures were identified from medical registry data. After correcting for age, body mass index (BMI), lifestyle factors and medication use, individuals with MS had lower bone width (β = -0.054, P = 0.003), lower cortical buckling ratio (β = -0.81, P = 0.003) and lower odds of having osteoporosis (odds ratio =0.56, P = 0.007) in women but not in men. Similarly, MS was associated with higher FN-BMD only in women (β = 0.028, P=0.001). In the analyses of MS components, the glucose component (unrelated to diabetes status) was positively associated with FN-BMD in both genders (β = 0.016, P = 0.01 for women and β = 0.022, P = 0.004 for men). In men, waist circumference was inversely associated with FN-BMD (β = -0.03, P = 0.004). No association was observed with fracture risk in either sex. In conclusion, women with MS had higher FN-BMD independent of BMI. The glucose component of MS was associated with high FN-BMD in both genders, highlighting the need to preserve glycemic control to prevent skeletal complications.

INTRODUCTION

Osteoporosis, a systemic skeletal disease, is an important public health problem due to its in-creased risk for fractures, high morbidity and mortality and significant health care costs.[1, 2] Different factors have been associated with osteoporosis including abdominal obesity, hypertension, dyslipidemia and abnormal glucose metabolism, which are considered components of metabolic syndrome. These components might affect bone differently. Obesity may lead to increased bone mineral density (BMD) because it is associated with higher 17β-estradiol levels and higher mechanical load, which may protect bone.[3,4] Visceral fat accumulation is associated with higher levels of proinflammatory cytokines, which may upregulate receptor activators of nuclear kappa B ligand, leading to increased bone resorption and therefore decreased BMD. [5-7] Also, although hyperglycemia is a predictor of osteoporotic fractures,[8] the association between high glucose levels or insulin resistance with BMD is not well defined. For example, diabetic individuals have higher BMD but increased risk of fractures.[9-11] Similarly, the evidence for associations between dyslipidemia and hypertension with bone metabolism is still inconclusive and the overall association between MS and bone health remains unclear. [12-15] Two recent meta-analyses on the association between MS and BMD and fractures, were not conclusive and whether metabolic syndrome might be associated with bone health beyond the contribution of the individual components remains unclear.[16,17] Moreover, sex-differences have been suggested in this relationship, mainly due to differences in body fat distribution between men and women, but yet not clearly defined.[18-20] Recently, a new cluster of criteria for diagnosis of MS had been presented with an emphasis on gender and ethnic differences in the measure of central obesity, [21] which has not been adequately studied in relation to bone.

We aimed to study the association between MS, femoral neck BMD (FN-BMD), hip bone geometry (HBG), osteoporosis, and fractures among elderly Dutch men and women, participants of the Rotterdam Study and if these associations were independent of body mass index (BMI). We examined whether MS was associated with FN-BMD and HBG parameters in females and males using a cross-sectional design and to determine whether MS predicts FN-BMD, and incident fractures using a longitudinal design.

MATERIALS AND METHODS

Study Population

The study was performed within the framework of the Rotterdam Study, a population-based cohort among persons ≥ 55 years and older living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of the Rotterdam Study is

described elsewhere. [22] The study was approved by the Medical Ethical Committee of Erasmus University, and all participants gave informed consent. The present study used data from the baseline examination of the third wave of the first cohort (RS-I-3) (1997–1999), in which 2,463 females and 1750 males participated and FN-BMD was measured at the fourth round (RSI-4) (2002–2004).

Population for Analysis

Metabolic Syndrome, FN-BMD, Hip Bone Geometry and Osteoporosis

Among 2463 females and 1750 males, 402 women and 231 men did not have fasting samples or measures of at least on component of MS, and were therefore excluded from the analysis. Furthermore, FN-BMD at RSI-3 (1997–1999), was not assessed in 534 females and 353 males, leaving 1527 females and 1166 males for the cross-sectional analysis regarding MS, FN-BMD and osteoporosis.For the analysis concerning MS and HBG, 124 female and 48 males were further excluded because of noavailable information on HBG data (**Supplementary Figure 1 and 2**).

Metabolic Syndrome and Fractures Risk

Among 2061 females and 1519 males with information available on MS components at RSI-3, 29 subjects (21 females and 9 males) were excluded because there was no data on fracture follow-up, hence leaving 2040 females and 1510 males for the analysis on MS and fracture risk. Furthermore, for each type of fracture, prevalent cases were excluded from the analysis (**Supplementary Figure 1 and 2**).

Metabolic Syndrome Definition

MS was defined according to the new criteria announced by a joint scientific statement from the International Diabetes Federation (IDF), the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), World Heart Federation, International Atherosclerosis Society and International Association for the Study of Obesity. [21] Participants with three or more of the following components were classified as having MS: (1) abdominal obesity (waist circumference (WC) ≥ 102 cm for men or ≥ 88 cm for women); (2) high triglycerides (TG) (≥ 150 mg/dL) (3) low HDL (≤ 40 mg/dL for men or ≤ 50 mg/dL for women) (4) elevated blood pressure (BP) (systolic BP ≥ 130 and/or diastolic BP ≤ 85 mmHg) or (5) high glucose (fasting glucose level ≥ 100 mg/dL). The criteria for abdominal obesity were adopted from the cut-offs for European people region. [21]

Skeletal Assessments

All events, including incident fractures and death, were reported by general practitioners (GPs) in the research area by means of a computerized system. All reported

events were verified by two trained research physicians, who independently reviewed and coded the information. Subsequently, all coded events were reviewed by a medical expert for final classification. Subjects were followed from their baseline visit until January 1, 2007 or until a first fracture or death occurred. FN-BMD (g/cm^2) at the RS1-3 (1997–1999) was measured by Dual-energy X-ray absorptiometry (DXA) using a Lunar DPX-L densitometer [23] (Lunar Radiation Corp., Madison, WI, USA) and analysed with DPX-IQ v.4.7d software whereas at the RS1-4 (2002–2004), FN-BMD was measured using a GE Lunar Prodigy bone densitometer. No cross-calibration between the two measures was per-formed. From the FN-BMD, sex-specific T-scores were calculated using the NHANES reference population. [24] Peak bone mass, as converted to the corresponding Lunar value, was 1.04 ± 0.14 g/cm^2 for women and 1.13 ± 0.16 g/cm^2 for men. Osteoporosis was defined as a T-score below -2.5 SD whereas osteopenia as a T-score between -1.0 and -2.5. Hip structural analysis [25] was used to measure HBG from the DXA scans of the femur narrow neck region as described previously. [26]

Assessment of Covariates

At the third visit, smoking habits were coded as current, and former/never. BMI was calculated as weight (in kg)/height (in m^2). Information on medication use at the third-round visit included the use of diuretics, hormonal replacement therapy, systemic corticosteroids, drugs for bone and other musculoskeletal diseases. A faller was defined as an individual with a history of one, two, or more falls without precipitating trauma (e.g., car accident or sport injury) in the 12 months preceding the interview at the third-round visit. Falling frequency was then recorded as never or at least one. At the third visit to the research center, the total weekly duration of physical activity was assessed by an adapted version of the Zutphen Physical Activity Questionnaire and the LASA Physical Activity Questionnaire. The Dutch Healthy Diet (DHD)-index assessed at the first wave of the Rotterdam Study (1989–1993), was used to take into account overall dietary quality. The DHD represents compliance to the Dutch Guidelines for a Healthy Diet as assessed from the FFQ at baseline. [27]

STATISTICAL ANALYSIS

Continuous variables were reported as mean ± SD unless stated otherwise and categorical variables were presented as percentages. Linear regression models and logistic regression were used to determine the cross-sectional association between MS and the number of MS components with FN-BMD, HBG parameters and osteoporosis. For the longitudinal association of MS and its components with FN-BMD, linear regression models were fitted in generalized estimated equations (GEE) with exchangeable

correlation structure adjusting for the within-subject correlations due to the repeated measurements of FN-BMD in the same individual (partial Pearson correlation = 0.93 and intra-class correlation = 0.96).[28] Risk of incident fractures was evaluated for the association with MS and the number of different MS components using Cox proportional hazard regression models. The proportional hazard assumption of the Cox model was checked by the visual inspection of log minus log plots and by performing an interaction test with time. Models for fracture, FN-BMD, and hip geometry were corrected for potential confounders, including age, height, smoking status, physical activity, alcohol intake, fallings in the last 12 months, use of diuretics drugs, use of hormone replacement therapy, use of cortico-steroids drugs, use of drugs for bone and other musculoskeletal diseases and Dutch Healthy Diet Index; cross-sectional FN-BMD and HBG were additionally adjusted for BMI and weight respectively, longitudinal analysis on FN-BMD were additionally adjusted for BMI and the two time points FN-BMD measurements were performed (index time) (to adjust also for the lack of cross-calibration between the two measures of BMD) and the interaction between MS (or MS component) and the index time whereas fracture analysis were further adjusted for weight. To analyse the relationship between individual features of MS and FN-BMD, GEE was performed after adjusting for the possible covariates described above with respect to the presence of each component. All analyses were conducted separately for men and women because of gender differences in MS and bone parameters (also the formal tests of interaction (sex×MS) in BMI adjusted models were statistically significant). To correct for multiple testing, a two-tailed P value of 0.025 or less was considered as statistically significant.

To examine if diabetic individuals could influence the associations, we reran all analyses excluding subjects with MS who had diabetes mellitus at the thirdround visit. To adjust for potential bias associated with missing data we used multiple imputation procedure (N = 5 imputations). Rubin's method was used for the pooled regression coefficients (β) and 95% Confidence Intervals. All analyses were performed using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

RESULTS

The baseline characteristics of the study population included in the analysis of MS with FN-BMD are shown in **Table 1**. Six hundred ninety-eight (45.7%) female and 435 (37.3%) male participants had MS. On average, female participants were older than men (72.38 vs. 72.04) and were more likely to have osteoporosis (18.2% vs. 14.8%). Anthropometric, lifestyle and other characteristics of the excluded participants did not substantially differ from the participants included in the study (data not shown).

Cross-sectional ssociation between Metabolic Syndrome, FN-BMD, Hip Bone Geometry and Osteoporosis

In females, in age-adjusted models, MS was associated with higher FN-BMD (β = 0.056,

Table 1 | Baseline characteristics for participants.

	Women (N = 1,527)	Men (N = 1,166)
Age	72.38 ±6.81	72.04 ±6.51
Metabolic Syndrome (n, %)	698 (45.7)	435 (37.3)
Bone mineral density, 1st round (g/cm^2)	0.82 ± 0.14	0.93 ± 0.14
Bone mineral density, 2ndround (g/cm^2)*	0.82 ± 0.13	0.92 ± 0.13
Cortical thickness (cm)**	0.13 ± 0.04	0.15 ± 0.03
Bone width (cm)**	2.93 ± 0.32	3.37 ± 0.30
Section modulus (cm^3)**	0.97 ± 0.31	1.41 ± 0.34
Cortical buckling ratio**	13.50 ± 5.00	13.15 ± 3.96
Osteoporosis (n, %)	278 (18.2)	172 (14.8)
Osteopenia (n, %)	855 (56.0)	640 (54.6)
Diabetes Mellitus (n, %)	194 (12.7)	172 (14.8)
BMI (kg/m^2)	27.15 ±4.25	26.35 ±3.19
Smoking (Yes) (n, %)	216 (14.0)	201 (17.2)
Physical activity (min/week)	2820.62 ±1103.74	2519.15 ±1178
Alcohol intake (g/day)	1.57 (396821.4)	4.29 (79364.3)
Dutch Healthy Diet-Index	50.84 ±9.92	45.55 ±9.78
Fallings in the last 12 months (n, %)	415 (27.2)	225 (19.3)
Diuretic drugs (n, %)	264 (17.3)	163 (14.0)
HRT (n, %)	66 (4.3)	3 (0.26)
Corticosteroid drugs (n, %)	51 (3.3)	25 (2.1)
Bone drugs (n, %)	56 (3.86)	6 (0.53)
Other musculoskeletal drugs (n, %)	32 (2.1)	6 (0.53)

HRT: Hormone replacement therapy
*999 female and 768 male individuals with available measure of BMD at the second round
**1,403 female and 1,118 male individuals with available measure of hip bone geometry

P = 4.5x10^{-17}), higher cortical thickness (β = 0.01, P = 6.2x10^{-7}), higher section modulus (β = 0.053, P = 0.001), lower cortical buckling ratio (β = -1.93, P = 2.2x10^{-11}) whereas no association was found with bone width (β = -0.033, P = 0.055) (**Table 2**). After additional adjustments for confounding by BMI, height, lifestyle factors and medication use, the associations were attenuated or reversed (**Table 2**); however MS was still positively associated with FN-BMD (β = 0.017, P = 0.010), bone width (β = -0.054, P = 0.003) and cortical buckling ratio (β = -0.81, P = 0.003) (**Table 2**). Similarly, in the multivariable models, the number of MS components was positively associated with FN-BMD (β = 0.006,P = 0.012), femoral neck width (β = -0.026, P<0.001) and cortical

Table 2 | The cross-sectional association of metabolic syndrome with bone mineral density and bone geometry.

Women			Men		
FN-BMD, n = 1,527			**FN-BMD, n = 1,166**		
Metabolic Syndrome	β (95%CI)	P	Metabolic Syndrome	β (95%CI)	P
Model 1	0.056 (0.043, 0.069)	$4.5x10^{-17}$	Model 1	0.031 (0.015, 0.047)	$7.4x10^{-5}$
Model 2	0.018 (0.003, 0.030)	0.007	Model 2	-0.005 (-0.014, 0.003)	0.55
Model 3	0.017 (0.004, 0.030)	0.1	Model 3	-0.002 (-0.020, 0.015)	0.82
No. of MS components			No. of MS components		
Model 1	0.022 (0.017, 0.026)	$8.08x10^{-20}$	Model 1	0.012 (0.006, 0.018)	$9.6x10^{-5}$
Model 2	0.007 (0.002, 0.011)	0.009	Model 2	-0.004 (-0.007, 0.001)	0.30
Model 3	0.006 (0.001, 0.011)	0.012	Model 3	-0.002 (-0.009, 0.005)	0.53
Cortical thickness, n = 1,403			**Cortical thickness, n = 1,118**		
Metabolic Syndrome	β (95%CI)	P	Metabolic Syndrome	β (95%CI)	P
Model 1	0.010 (0.005, 0.014)	$6.2x10^{-7}$	Model 1	0.009 (0.005, 0.012)	$9.3x10^{-7}$
Model 2	0.001 (-0.003, 0.005)	0.69	Model 2	-0.001 (-0.003, 0.001)	0.56
Model 3	0.001 (-0.004, 0.006)	0.73	Model 3	-0.0005 (-0.004, 0.003)	0.80
No. of MS components			No. of MS components		
Model 1	0.004 (0.003, 0.006)	$1.33x10^{-8}$	Model 1	0.003 (0.002, 0.005)	$7.3x10^{-7}$
Model 2	0.001 (-0.001, 0.002)	0.42	Model 2	-0.001 (-0.002, 0.000)	0.26
Model 3	0.001 (-0.001, 0.002)	0.49	Model 3	-0.001 (-0.002, 0.001)	0.45
Bone width, n = 1,403			**Bone width, n = 1,118**		
Metabolic Syndrome	β (95%CI)	P	Metabolic Syndrome	β (95%CI)	P
Model 1	-0.033 (-0.068, 0.001)	0.055	Model 1	0.006 (-0.003, 0.042)	0.76
Model 2	-0.051 (-0.09, -0.015)	0.005	Model 2	-0.030 (-0.068, 0.008)	0.12
Model 3	-0.054 (-0.091, -0.018)	0.003	Model 3	-0.029 (-0.068, 0.010)	0.14
No. of MS components			No. of MS components		
Model 1	-0.015 (-0.027, -0.002)	0.021	Model 1	0.001 (-0.014, 0.015)	0.93
Model 2	-0.025 (-0.038, -0.011)	0.0003	Model 2	-0.014 (-0.029, 0.001)	0.076
Model 3	-0.026 (-0.039, -0.012)	0.0002	Model 3	-0.013 (-0.029, 0.002)	0.097

Table 2 | The cross-sectional association of metabolic syndrome with bone mineral density and bone geometry. (*continued*)

	Women			Men	
	Section Modulus, n = 1,403		**Section Modulus, n = 1,118**		
Metabolic Syndrome	β (95%CI)	P	Metabolic Syndrome	β (95%CI)	P
Model 1	0.053 (0.021, 0.086)	0.001	Model 1	0.096 (0.056, 0.136)	$3.0x10^{-7}$
Model 2	-0.029 (-0.062, 0.003)	0.08	Model 2	-0.028 (-0.068, 0.011)	0.159
Model 3	-0.03 (-0.063, 0.003)	0.078	Model 3	-0.021 (-0.061, 0.019)	0.30
No. of MS components			No. of MS components		
Model 1	0.024 (0.012, 0.036)	$5.6x10^{-5}$	Model 1	0.036 (0.021, 0.052)	$5.0x10^{-7}$
Model 2	-0.011 (-0.023, 0.002)	0.09	Model 2	-0.017 (-0.032, -0.001)	0.036
Model 3	-0.011 (-0.023, 0.001)	0.08	Model 3	-0.014 (-0.030, 0.002)	0.094
	Cortical Buckling Ratio, n = 1,403			**Cortical Buckling Ratio, n = 1,118**	
Metabolic Syndrome	β (95%CI)	P	Metabolic Syndrome	β (95%CI)	P
Model 1	-1.93 (-2.42, -1.43)	$2.2x10^{-11}$	Model 1	-0.88 (-1.35, -0.41)	$2.2x10^{-4}$
Model 2	-0.79 (-1.32, -0.26)	0.004	Model 2	0.036 (-0.23, 03037)	0.89
Model 3	-0.81 (-1.34, -0.27)	0.003	Model 3	-0.049 (-0.56, 0.46)	0.85
No. of MS components			No. of MS components		
Model 1	-0.71 (-0.89, -0.53)	$3.1x10^{-12}$	Model 1	-0.314 (-0.50, -0.13)	0.001
Model 2	-0.25 (-0.45, -0.06)	0.012	Model 2	0.101 (-0.003, 0.21)	0.33
Model 3	-0.26 (-0.46, -0.06)	0.011	Model 3	0.07 (-0.14, 0.27)	0.51

MS. metabolic syndrome; FN-BMD, femoral neck bone mineral density

Model 1: Adjusted for age

Model 2: Model 1 +body mass index and height for FN-BMD and weight + height for hip bone geometry parameters.

Model 3: Model 2 + smoking status, physical activity, alcohol intake, fallings in the last 12 months. use of diuretics drugs, use of hormone replacement therapy, use of corticosteroids drugs, use of drugs for bone and other musculoskeletal diseases and Dutch Healthy Diet Index.

buckling ratio (β = -0.26, P = 0.011) whereas no association was observed between MS and cortical thickness or section modulus in females (P>0.025) (**Table 2**). Also, MS and the number of its components, were inversely associated with osteopenia and osteoporosis in age-adjusted models (osteopenia: OR = 0.65, P = $3.2x10^{-4}$, P *for no. of MS components*<0.0001; osteoporosis: OR = 0.23, P = $3.3x10^{-16}$, P *for no. of MS components*<0.0001) (**Figure 1**). After adjustment for BMI although attenuated, the inverse association between MS and osteoporosis remained significant (OR = 0.56, P = 0.006) (**Figure 1**). Also, a positive association was observed between the number of MS features and osteoporosis in the multivariable model P *for no. of MS components* = 0.03) (**Figure 1**). Additional adjustment for other confounders did not affect the results (data not shown). In males, MS was associated with higher FN-BMD (β = 0.031, P = $7.4x10^{-5}$), higher cortical thickness (β = 0.009, P = $9.3x10^{-7}$), higher section modulus (β = 0.096, P = $3.0x10^{-7}$) and

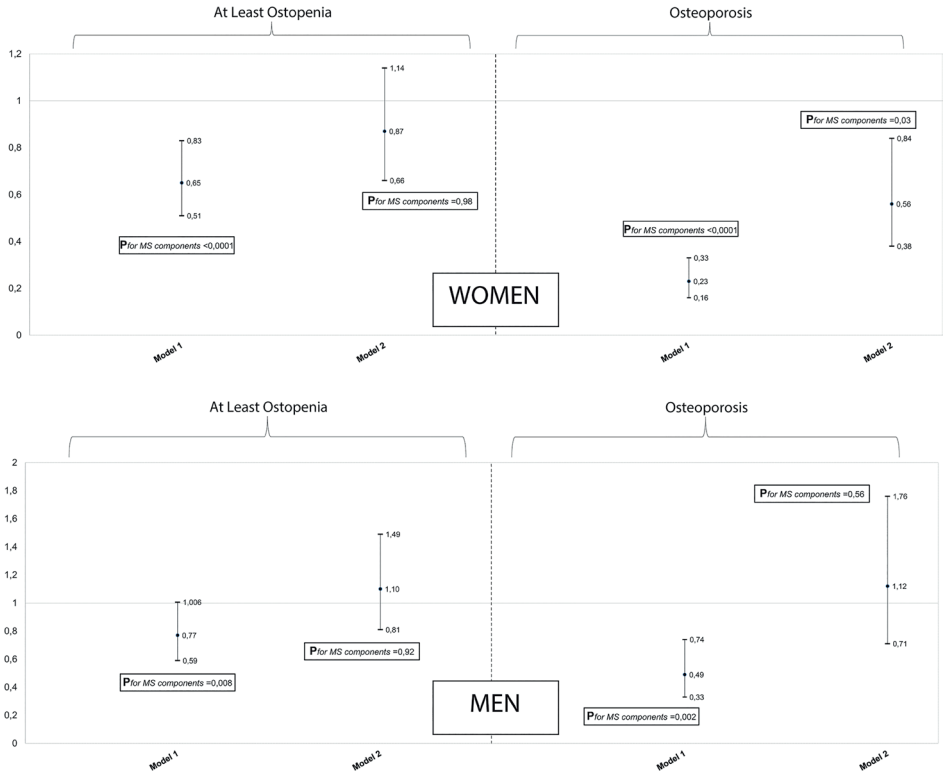


Figure 1 | The association between metabolic syndrome, osteopenia and osteoporosis in women and men.

Reference group are subjects with no osteopenia, neither osteoporosis: Confounders include age, body mass index, height, smoking status, physical activity, alcohol intake, fallings in the last 12 months, use of diuretics drugs, use of hormone replacement therapy, use of corticosteroids drugs, use of drugs for bone and other musculoskeletal diseases and Dutch Healthy Diet Index.

lower cortical buckling ratio (β = -0.88, P = $2.2x10^{-4}$) in age-adjusted models (**Table 2**). However, after adjustment for BMI and height, MS was neither statistically significantly assossiated with FN-BMF nor with hip bome geometry parameters (**Table 2**). Similarly, there was no association between number of MS components, FN-BMD and hip bone geometry in the fully adjusted models (**Table 2**). Also, in age-adjusted models, MS was inversely associated with osteoporosis but not with osteopenia (osteopenia: OR = 0.77, P = 0.056, $P_{for\ no.\ of\ MS\ components}$ = 0.008; osteoporosis: OR = 0.49, P = 0.001, $P_{for\ no.\ of\ MS\ components}$ = 0.002) (**Figure 1**). However, no association was observed between MS and its components with osteopenia or osteoporosis after adjustment for BMI (**Figure 1**) and other confounders (data not shown).

Longitudinal association between Metabolic Syndrome and FN-BMD

Similar to the cross-sectional analysis, MS and the number of its features was positively associated with FN-BMD (β = 0.028, P = 0.001; $P_{for\ no.\ of\ MS\ components}$ = 0.001) in the

Table 3 | The longitudinal association of metabolic syndrome with bone mineral density.

Women (N = 1,527)			Men (N = 1,166)#		
Metabolic syndrome (Yes vs. No)	**FN-BMD**	**P**	**Metabolic syndrome (Yes vs. No)**	**FN-BMD**	**P**
Model 1: β. 95% CI	0.063 (0.048, 0.079)	$2.44x10^{-15}$	Model 1: β. 95% CI	0.031 (0.015, 0.047)	0.0001
Model 2: β. 95% CI	0.028 (0.015, 0.042)	0.001	Model 2: β. 95% CI	-0.006 (-0.016, 0.004)	0.58
Model 3: β. 95% CI	0.028 (0.012, 0.043)*	0.001	Model 3: β. 95% CI	-0.002 (-0.022, 0.017)	0.83
No. of MS components (continuous)			**No. of MS components (continuous)**		
Model 1: β. 95% CI	0.025 (0.019, 0.030)	$5.4x10^{-20}$	Model 1: β. 95% CI	0.013 (0.006, 0.020)	0.0003
Model 2: β. 95% CI	0.011 (0.005, 0.016)	0.0004	Model 2: β. 95% CI	-0.003 (-0.007, 0.001)	0.46
Model 3: β. 95% CI	0.010 (0.004, 0.016)**	0.001	Model 3: β. 95% CI	-0.001 (-0.009, 0.006)	0.75

MS, metabolic syndrome; FN-BMD, femoral neck bone mineral density
Model 1: Adjusted for age and type of DXA scan
Model 2: Model 1 +body mass index and height
Model 3: Model 2 + smoking status, physical activity, alcohol intake, fallings in the last 12 months, use of diuretics drugs, use of hormone replacement therapy, use of corticosteroids drugs, use of drugs for bone and other musculoskeletal diseases and Dutch Healthy Diet Index.
*index time (time points when the DXA measurements were performed), β = -0.012. p<0.001; interaction MS x index time: β = -0.008, p = 0.031
** index time, β = -0.012. p<0.001; interaction MS component x index time: β = -0.003, p = 0.021
#no significant interaction between MS (or MS component) and index time (p>0.50) was observed in any of the analysis in men and therefore data are not shown

multivariable model in females, which tended to go away across time (interaction MS x index time: β = -0.008, p = 0.031; interaction MS component x index time: β = -0.003, p = 0.021). No association was observed between MS or its features and adjusted FN-BMD in males (**Table 3**).

Association between Metabolic Syndrome and Fracture Risk

In females, no association was observed between MS and any type of fractures, neither in age and gender adjusted model, nor in the multivariable model (HR = 0.91: 95%CI: 0.73–1.15) (**Table 4**). Also no association was observed for non-vertebral fractures (HR = 0.94: 95%CI: 0.73–1.21) or vertebral fractures (HR = 0.83: 95%CI: 0.56–1.24) (**Table 3**). Similarly, there was no significant association between MS with any type of fractures or with subtypes of fractures in males (**Table 4**). Nevertheless, in males, significant inverse associations were observed between numbers of MS components with any type of fractures (P = 0.015) and with non-vertebral fractures (P = 0.017) (**Table 3**).

Additional Analysis

Supplementary Table 1 shows the effect of individual components of MS on the adjusted FN-BMD. In both genders, elevated glucose levels were associated with higher BMD (in females: β = 0.016, P = 0.01; in males: β = 0.022, P = 0.004). In women, but not in men, HDL-cholesterol was positively associated with FN-BMD (β = 0.013, P = 0.01). In men but not in women, waist circumference component was inversely associated with FN-BMD (in males: β = -0.030, P = 0.004). No association was observed between other components of MS and adjusted FN-BMD in either gender.

Exclusion of subjects with MS having diabetes mellitus (194 females and 172 males) did not substantially change any of the results (data not shown). Moreover, substitution in the multivariable models of BMI with weight and vice versa, did not change any of the results (data not shown).

DISCUSSION

To our knowledge, this is the first study to simultaneously examine the association between metabolic syndrome, FN-BMD, HBG parameters, osteoporosis and fracture risk by using the most recent definition of MS. Females with MS, have significantly higher BMD, narrower bone at the hip, and increased bone instability (lower buckling ratio), lower odds of having osteoporosis than non-MS individuals, independent of BMI. In males, the positive association of MS with BMD and HBG geometry was explained

Table 4 | Metabolic syndrome and fracture risk.

Women			Men		
All Fractures (371)			**All Fractures (147)**		
Metabolic Syndrome	Hazard ratio (95%CI)	P	Metabolic Syndrome	Hazard ratio (95%CI)	P
Model 1	0.85 (0.69–1.04)	0.12	Model 1	0.85 (0.60–1.20)	0.36
Model 2	0.91 (0.72–1.14)	0.40	Model 2	0.74 (0.69–1.08)	0.12
Model 3	0.91 (0.73–1.15)	0.43	Model 3	0.68 (0.46–1.006)	0.054
No. of MS components			No. of MS components		
Model 1	0.94 (0.87–1.02)	0.11	Model 1	0.92 (0.81–1.06)	0.24
Model 2	0.97 (0.89–1.05)	0.44	Model 2	0.86 (0.74–1.001)	0.055
Model 3	0.97 (0.89–1.06)	0.47	Model 3	0.82 (0.70–0.9964)	0.015
Non-Vertebral Fractures (307)			**Non-Vertebral Fractures (102)**		
Metabolic Syndrome	Hazard ratio (95%CI)	P	Metabolic Syndrome	Hazard ratio (95%CI)	P
Model 1	0.90 (0.72–1.13)	0.36	Model 1	0.89 (0.59–1.35)	0.59
Model 2	0.94 (0.73–1.21)	0.63	Model 2	0.69 (0.55–1.88)	0.12
Model 3	0.94 (0.73–1.21)	0.61	Model 3	0.64 (0.40–1.03)	0.068
No. of MS components			No. of MS components		
Model 1	0.96 (0.89–1.05)	0.38	Model 1	0.94 (0.79–1.10)	0.42
Model 2	0.98 (0.89–1.08)	0.70	Model 2	0.83 (0.68–1.01)	0.047
Model 3	0.98 (0.89–1.08)	0.66	Model 3	0.75 (0.66–0.96)	0.017
Vertebral Fractures (123)			**Vertebral Fractures (62)**		
Metabolic Syndrome	Hazard ratio (95%CI)	P	Metabolic Syndrome	Hazard ratio (95%CI)	P
Model 1	0.68 (0.47–0.98)	0.039	Model 1	0.65 (0.37–1.13)	0.13
Model 2	0.80 (0.53–1.19)	0.27	Model 2	0.67 (0.49–1.25)	0.20
Model 3	0.83 (0.56–1.24)	0.36	Model 3	0.60 (0.32–1.14)	0.12
No. of MS components			No. of MS components		
Model 1	0.84 (0.73–0.96)	0.01	Model 1	0.84 (0.60–1.03)	0.10
Model 2	0.86 (0.76–1.03)	0.11	Model 2	0.85 (0.75–1.07)	0.17
Model 3	0.90 (0.77–1.05)	0.18	Model 3	0.81 (0.64–1.03)	0.09

"( )", number of fractures
Model 1: Adjusted for age
Model 2: Model 1 +Height and Weight
Model 3: Model 2 + smoking status, physical activity, alcohol intake, fallings in the last 12 months, use of diuretics drugs, use of hormone replacement therapy, use of corticosteroids drugs, use of drugs for bone and other musculoskeletal diseases and Dutch Healthy Diet Index.

by BMI. Our results on fracture risk, suggest no consistent influence of MS in either gender.

According to the observational data in the past decade, the relationship between MS and its components, BMD and fracture risk is controversial. In line with our findings, a recent meta-analysis showed that MS may have beneficial influence on BMD in Caucasian individuals.[16] Also, another meta-analysis on MS and fracture risk, concluded that individuals with MS are not at higher risk of having fractures [17]. MS is a cluster of conditions, interacting with each other, and therefore the mechanism behind the effect of MS on BMD and fractures risk is complicated and has not yet been investigated in detail. Although, the association between individual components of MS and bone metabolism have been extensively studied, the results are yet inconclusive. (1) For example, central obesity has been associated with higher BMD in some studies, but some others and our study as well have shown detrimental effects [29,30]. Also, obesity, is a risk factor for fractures of the humerus and ankle, but protects against fractures of hip and vertebral bodies.[31-33] (2) Elevated glucose levels have been linked to better, worse or similar bone outcomes.[18,34,35] (3) Hypertension is postulated to be associated with low bone mass due to urinary calcium excretion and therefore increase fracture risk,[36] but, contrary to common belief, intensive antihypertensive treatment was not associated with an increased risk of falls or non-spine fractures in patients with type 2 diabetes in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) randomized trial.[37] Hypertriglyceridemia contributes to a lower risk of fractures, which may partly be explained by the interaction with protein matrix and bone minerals.[12,38] However, Kim et al[39] and Adami et al[13] found that high triglyceride levels and low HDL-cholesterol were negatively associated with BMD. Therefore, as observed in the current study, the combined effects of these components on bone metabolism may be beneficial or insignificant.

The positive association between MS and BMD observed in the present study was mainly driven by glucose levels. MS is a risk factor for type 2 diabetes mellitus which has been associated with an increase in fracture risk. In contrast to MS individuals, evaluation of BMD may not be adequate for predicting fracture risk in patients with type 2 diabetes, who are unlikely to be diagnosed with osteoporosis and increased risk of fracture.[10] In the large Rotterdam Study, we previously showed that, similar to MS individuals, diabetic patients have higher BMD and stronger bone geometry, which would protect them against fractures.[10] However, in contrast to MS individuals, diabetic individuals have an increased fracture risk, which was shown to be driven by poor glycemic control.[10] We postulate that increased bone fragility may be caused by chronically elevated glucose levels, which may lead to accumulation of microcracks and/or cortical porosity. MS is a health condition which predisposes to type 2 diabetes mellitus, but may not yet be characterized by chronically elevated glucose levels.

In the present study, the positive association between MS and BMD persisted also after exclusion of subjects with MS who had diabetes mellitus. Thus, it is likely that, microcracks and/or cortical porosity may not be present in MS individuals which may explain why MS subjects do not yet experience a high risk of fractures (**Figure 2**). Follow-up studies focusing on the health life trend of MS individuals in relation to bone are thus needed.

The current study shows that no gender differences are observed between MS and unadjusted BMD and fracture risk, but that gender differences become prominent after adjustment for BMI and that in men, the observed positive association between MS and BMD and hip bone geometry parameters is explained by obesity. Similar to our observations, the meta-analysis investigating the association between MS and unadjusted BMD concluded that there were no gender differences.[16] Also, the recent meta-analysis examining the association between MS and fracture risk did not observed any difference between men and women with similar estimates to our study.[17] In contrast, gender differences in the association between MS and BMI-adjusted BMD have been

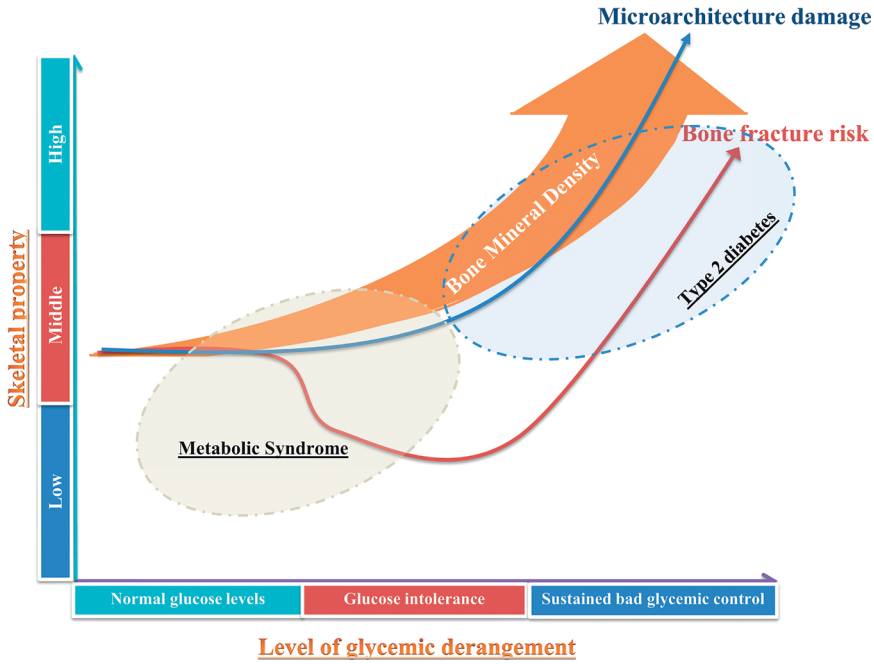


Figure 2 | Level of glycemic derangement, bone architecture and fracture risk.
Cartoon depicting the differences in bone mineral density, fracture risk and changes in bone microarchitecture across the stages of glucose derangement. Metabolic syndrome and diabetes mellitus individuals have higher BMD but do not experience yet an increase in fracture risk. With sustained bad glycemic control, the damage of bone microarchitecture represented by accumulation of microcracks and cortical porosity becomes a possibility which may explain the bone fragility and fracture susceptibility despite the observed increase in BMD. Drawing is not to scale.

previously described, showing less beneficial effects of MS on bone health in men than in women. [39,40] In contrast to our results, both studies observed a decrease in FN-BMD with increasing number of MS components in men, but not in women. However, both studies were characterized by other ethnicities or were performed in a younger population. It has been shown that the association between MS and BMD differs by ethnicity.[16] Also, BMD declines by age and this decline differs by gender.[41] In a twin study, fat mass and body fat distribution seem to have different relationships with BMD according to gender and age.[42] Since fat deposition differs according to gender, it has been hypothesized that mechanical effect and estrogen synthesis predominate in women, while bone-deleterious effects of fat, related to oxidative stress and chronic inflammation, predominate in men.[43] Therefore, bone mass in males with MS may be more influenced by visceral fat than in women which may explain the dilution of the association after adjustment for BMI and the relationship between waist circumference and BMD in men and not in women.

There are several strengths of our present study. First, this is a large prospective population-based study of 3,458 individuals, with comprehensive follow up of more than 6.7 years on aver-age. We were also able to examine both cross-sectional and longitudinal the associations between MS, BMD and osteoporosis. Additionally, the present study had various indices of bone outcomes available, including hip bone geometry parameters and fracture incidence. Moreover, in contrast to other studies, we used the most recent definition of MS. Yet our study has some limitations. We did not have measures of glucose control, such as HbA1c which could have strengthened our results. Secondly, the subjects in this study were only Caucasians. Ethnic differences in the association between MS and/or its components and bone have been previously reported. Thus, our findings may not be extended to non-Caucasian groups.

In conclusion, MS is associated with higher BMD, increase instability and narrower bone, which is mainly driven by elevated glucose levels. In men but not in women, higher FN-BMD was mainly explained by body mass index and body fat distribution. We postulate that the bone of MS individuals is not yet characterized by an accumulation of microcracks (cortical porosity) that would reflect sustained impairment of bone structure, and therefore yet an increased risk of fragility, as observed in diabetic individuals. These highlight the importance of maintaining glycemic control in individuals with MS to pervert skeletal complications and preserve bone health.

Detailed acknowledgments and online resources can be found in the published article online

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0129116

REFERENCES

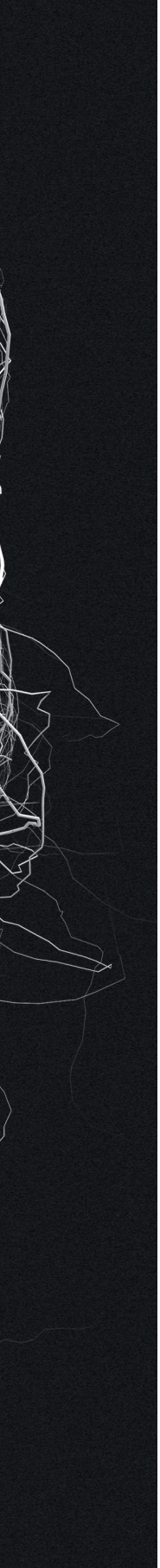
1. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR (2009) Mortality Risk Associated With Low-Trauma Osteoporotic Fracture and Subsequent Fracture in Men and Women. Jama-Journal of the American Medical Association 301: 513–521. doi: 10.1001/jama.2009.50 PMID: 19190316
2. Center JR, Nguyen TV, Schneider D, Sambrook PN, Eisman JA (1999) Mortality after all major types of osteoporotic fracture in men and women: an observational study. LANCET 353: 878–882. PMID: 10093980
3. Nelson LR, Bulun SE (2001) Estrogen production and action. Journal of the American Academy of Dermatology 45: S116–S124. PMID: 11511861
4. Ohta H, Ikeda T, Masuzawa T, Makita K, Suda Y, Nozawa S (1993) Differences in Axial Bone-Mineral Density, Serum Levels of Sex Steroids, and Bone Metabolism between Postmenopausal and Age- Matched and Body Size-Matched Premenopausal Subjects. Bone 14: 111–116. PMID: 8334027
5. Hofbauer LC, Schoppet M (2004) Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. Jama-Journal of the American Medical Association 292: 490–495.
6. Smith BJ, Lerner MR, Bu SY, Lucas EA, Hanas JS, Lightfoot SA, et al. (2006) Systemic bone loss and induction of coronary vessel disease in a rat model of chronic inflammation. Bone 38: 378–386. PMID: 16256450
7. Campos RMS, de Piano A, da Silva PL, Carnier J, Sanches PL, Corgosinho FC, et al. (2012) The role of pro/anti-inflammatory adipokines on bone metabolism in NAFLD obese adolescents: effects of longterm interdisciplinary therapy. Endocrine 42: 146–156. PMID: 22315014
8. Janghorbani M, Van Dam RM, Willett WC, Hu FB (2007) Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol 166: 495–505. PMID: 17575306
9. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. (2011) Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA 305: 2184– 2192. doi: 10.1001/jama.2011.715 PMID: 21632482
10. Oei L, Zillikens MC, Dehghan A, Buitendijk GHS, Castano-Betancourt MC, Estrada K, et al. (2013) High Bone Mineral Density and Fracture Risk in Type 2 Diabetes as Skeletal Complications of Inadequate Glucose Control The Rotterdam Study. Diabetes Care 36: 1619–1628. doi: 10.2337/dc12-1188 PMID: 23315602 Metabolic Syndrome and Bone Health PLOS ONE | DOI:10.1371/journal.pone.0129116 June 12, 2015 13 / 15
11. Ma LL, Oei L, Jiang LD, Estrada K, Chen HY, Wang Z, et al. (2012) Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. European Journal of Epidemiology 27: 319–332. doi: 10.1007/s10654-012-9674-x PMID: 22451239
12. Yamaguchi T, Sugimoto T, Yano S, Yamauchi M, Sowa H, Chen QX, et al. (2002) Plasma lipids and osteoporosis in postmenopausal women. Endocrine Journal 49: 211–217. PMID: 12081241
13. Adami S, Braga V, Zamboni M, Gatti D, Rossini M, Bakri J, et al. (2004) Relationship between lipids and bone mass in 2 cohorts of healthy women and men. Calcified Tissue International 74: 136–142. PMID: 14668965

14. Tsuda K, Nishio I, Masuyama Y (2001) Bone mineral density in women with essential hypertension. American Journal of Hypertension 14: 704–707. PMID: 11465657
15. Cappuccio FP, Meilahn E, Zmuda JM, Cauley JA, Grp SOFR (1999) High blood pressure and bonemineral loss In elderly white women: a prospective study. LANCET 354: 971–975. PMID: 10501357
16. Xue P, Gao P, Li YK (2012) The association between metabolic syndrome and bone mineral density: a meta-analysis. Endocrine 42: 546–554. doi: 10.1007/s12020-012-9684-1 PMID: 22547367
17. Sun K, Liu JM, Lu N, Sun HX, Ning G (2014) Association between metabolic syndrome and bone fractures: a meta-analysis of observational studies. Bmc Endocrine Disorders 14.
18. Yamaguchi T, Kanazawa I, Yamamoto M, Kurioka S, Yamauchi M, Yano S, et al. (2009) Associations between components of the metabolic syndrome versus bone mineral density and vertebral fractures in patients with type 2 diabetes. Bone 45: 174–179. doi: 10.1016/j.bone.2009.05.003 PMID: 19446053
19. BarrettConnor E, KritzSilverstein D (1996) Does hyperinsulinemia preserve bone? DIABETES CARE 19: 1388–1392. PMID: 8941469
20. Kim H, Oh HJ, Choi H, Choi WH, Lim SK, Kim JG (2013) The association between bone mineral density and metabolic syndrome: a Korean population-based study. J Bone Miner Metab 31: 571–578. doi: 10. 1007/s00774-013-0446-9 PMID: 23529801
21. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. (2009) Harmonizing the Metabolic Syndrome A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120: 1640–1645. doi: 10.1161/CIRCULATIONAHA.109.192644 PMID: 19805654
22. Hofman A, Murad SD, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, et al. (2013) The Rotterdam Study: 2014 objectives and design update. European Journal of Epidemiology 28: 889–926. doi: 10.1007/s10654-013-9866-z PMID: 24258680
23. Burger H, de Laet CEDH, van Daele PLA, Weel AEAM, Witteman JCM, Hofman A, et al. (1998) Risk factors for increased bone loss in an elderly population—The Rotterdam Study. American Journal of Epidemiology 147: 871–879. PMID: 9583718
24. Looker AC, Wahner HW, Dunn WL, Calvo MS, Harris TB, Heyse SP, et al. (1998) Updated data on proximal femur bone mineral levels of US adults. Osteoporosis International 8: 468–489. PMID: 9850356
25. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW (2000) Structural trends in the aging femoral neck and proximal shaft: Analysis of the Third National Health and Nutrition Examination Survey dualenergy X-ray absorptiometry data. Journal of Bone and Mineral Research 15: 2297–2304. PMID: 11127194
26. Rivadeneira F, Zillikens MC, De Laet CE, Hofman A, Uitterlinden AG, Beck TJ, et al. (2007) Femoral neck BMD is a strong predictor of hip fracture susceptibility in elderly men and women because it detects cortical bone instability: The Rotterdam study. Journal of Bone and Mineral Research 22: 1781– 1790. PMID: 17638578
27. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, Geleijnse JM, Hofman A, Grobbee DE, et al. (1998) Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. Eur J Clin Nutr 52: 588–596. PMID: 9725660

28. Zeger SL, Liang KY, Albert PS (1988) Models for Longitudinal Data—a Generalized Estimating Equation Approach. Biometrics 44: 1049–1060. PMID: 3233245
29. Edelstein SL, Barrettconnor E (1993) Relation between Body-Size and Bone-Mineral Density in Elderly Men and Women. American Journal of Epidemiology 138: 160–169. PMID: 8356959
30. Jankowska EA, Rogucka E, Medras M (2001) Are general obesity and visceral adiposity in men linked to reduced bone mineral content resulting from normal ageing? A population-based study. Andrologia 33: 384–389. PMID: 11736801 Metabolic Syndrome and Bone Health PLOS ONE | DOI:10.1371/journal.pone.0129116 June 12, 2015 14 / 15
31. Schott AM, Cormier C, Hans D, Favier F, Hausherr E, Dargent-Molina P, et al. (1998) How hip and whole-body bone mineral density predict hip fracture in elderly women: The EPIDOS prospective study. Osteoporosis International 8: 247–254. PMID: 9797909
32. Gnudi S, Sitta E, Lisi L (2009) Relationship of body mass index with main limb fragility fractures in postmenopausal women. Journal of Bone and Mineral Metabolism 27: 479–484. doi: 10.1007/s00774-009- 0056-8 PMID: 19277453
33. Spaine LA, Bollen SR (1996) 'The bigger they come. . .': The relationship between body mass index and severity of ankle fractures. Injury-International Journal of the Care of the Injured 27: 687–689. PMID: 9135744
34. Kinjo M, Setoguchi S, Solomon DH (2007) Bone mineral density in adults with the metabolic syndrome: Analysis in a population-based US sample. Journal of Clinical Endocrinology & Metabolism 92: 4161– 4164.
35. Holmberg AH, Nilsson PM, Nilsson JA, Akesson K (2008) The association between hyperglycemia and fracture risk in middle age. A prospective, population-based study of 22,444 men and 10,902 women. Journal of Clinical Endocrinology & Metabolism 93: 815–822.
36. Vestergaard P, Rejnmark L, Mosekilde L (2009) Hypertension Is a Risk Factor for Fractures. Calcified Tissue International 84: 103–111. doi: 10.1007/s00223-008-9198-2 PMID: 19067019
37. Margolis KL, Palermo L, Vittinghoff E, Evans GW, Atkinson HH, Hamilton BP, et al. (2014) Intensive Blood Pressure Control, Falls, and Fractures in Patients with Type 2 Diabetes: The ACCORD Trial. J Gen Intern Med.
38. Xu SH, Yu JQJ (2006) Beneath the minerals, a layer of round lipid particles was identified to mediate collagen calcification in compact bone formation. Biophysical Journal 91: 4221–4229. PMID: 16980361
39. Kim HY, Choe JW, Kim HK, Bae SJ, Kim BJ, Lee SH, et al. (2010) Negative Association between Metabolic Syndrome and Bone Mineral Density in Koreans, Especially in Men. Calcified Tissue International 86: 350–358. doi: 10.1007/s00223-010-9347-2 PMID: 20354685
40. Kim H, Oh HJ, Choi H, Choi WH, Lim SK, Kim JG (2013) The association between bone mineral density and metabolic syndrome: a Korean population-based study. Journal of Bone and Mineral Metabolism 31: 571–578. doi: 10.1007/s00774-013-0446-9 PMID: 23529801
41. Krall EA, DawsonHughes B, Hirst K, Gallagher JC, Sherman SS, Dalsky G (1997) Bone mineral density and biochemical markers of bone turnover in healthy elderly men and women. Journals of Gerontology Series a-Biological Sciences and Medical Sciences 52: M61–M67.

42. Makovey J, Naganathan V, Sambrook P (2005) Gender differences in relationships between body composition components, their distribution and bone mineral density: a cross-sectional opposite sex twin study. Osteoporosis International 16: 1495–1505. PMID: 15838718
43. Hernandez JL, Olmos JM, Gonzalez-Macias J (2011) Metabolic syndrome, fractures and gender. Maturitas 68: 217–223. doi: 10.1016/j.maturitas.2010.12.010 PMID: 21251772





2.1.3

The Association between Osteocalcin, Adiposity and Bone health: The Rotterdam Study

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ABSTRACT

Osteocalcin, an indicator of osteoblast differentiation, is an emerging bone turnover marker. Recently, there has been a growing interest in the potential extra-skeletal effects of osteocalcin such as regulation of glucose and energy metabolism; important predictors of bone mineral density (BMD). Therefore, we aimed to investigate the association between osteocalcin, adiposity and BMD. We included 6,619 participants (55.8% female) from the Rotterdam Study a prospective population-based cohort with mean age of 62.6±9.1. All participants underwent Dual Energy X-Ray Absorptiometry (DXA) to measure femoral neck (FN) BMD. Incident fractures were identified and collected from medical registry data. Different anthropometric and DXA-derived parameters were used as proxies for adiposity. Serum osteocalcin was measured by electrochemiluminescence immunoassay (ECLIA). Linear regression was performed adjusted for different confounders to test the association between mean osteocalcin levels with BMD and adiposity. Next, we used a time-to-event approach to estimate hazard ratios of sustaining future fracture. Our results showed strong inverse associations between osteocalcin and FN-BMD (β: -0.026; 95%CIs: -0.029 to -0.022) and Body mass index (BMI) (β: -0.919; 95%CIs: -1.093 to -0.745) in both males and females. Osteocalcin was also inversely associated with fat mass, waist circumference, and other measurements of adiposity. Individuals with low BMI and high osteocalcin levels had significantly higher prevalence of osteopenia and osteoporosis (60.2% and 12.6%) compared to individuals with BMI and osteocalcin levels in the middle range (46.4% and 4.3%). In addition, higher levels of osteocalcin were associated with increased risk of wrist fractures (HR=1.30; 95%CIs: 1.13 to 1.49) but not hip fractures (HR: 0.95; 95%CIs: 0.82 to 1.10). In conclusion, the presence of low BMI and high osteocalcin is an indication of seriously declined bone health, corroborated by the high prevalence of osteoporosis and the subsequent risk of wrist fractures.

INTRODUCTION

For a long time osteocalcin was considered to be a bone-specific protein involved in the process of bone mineralization; emerging as a prominent serum marker of osteoblastic bone formation in clinical practice.[1] Although, the majority of the osteocalcin is absorbed by the bone hydroxyapatite, around 10-20% of the total amount is released into the circulation[2] exerting significant effects on fat and global energy metabolism.[3,4]

The inverse relationship between serum osteocalcin levels and bone mineral density (BMD) is well documented in postmenopausal women.[5,6,7,8,9] In addition, it has been suggested that higher levels of osteocalcin are a marker of hip fractures in elderly women.[10,11] In the past few years, there has been growing evidence of the extra-skeletal effects of osteocalcin, demonstrating its inverse associations with fat mass, lipid metabolism, body mass index (BMI) and other measurements of adiposity.[12,13,14,15,16] BMI, a proxy of adiposity, is a relevant risk factor for osteoporosis and fracture risk[17,18] whose mechanisms of action go beyond mechanical loadings.[19,20] The adipose tissue secretes various factors such as adiponectin, leptin and adipokines, which may affect osteoblast differentiation and bone formation.[21,22] High adiposity has been also associated with increased insulin resistance which can suppress bone turnover and subsequently decrease osteocalcin levels.[23] Hence, adiposity may be an important mediator of the relationship between osteocalcin and BMD.

To our knowledge, there are no studies that have specifically investigated the joint effect of osteocalcin and adiposity on bone health. In addition, limited evidence exists on the effect of osteocalcin on BMI, BMD, and fracture risk in men. Therefore, to further understand the complex relationship between osteocalcin, adiposity and bone health, we sought to study 1) the joint effect of osteocalcin and adiposity on bone and 2) whether adiposity is a mediator of the association of osteocalcin with BMD in a large population-based cohort of elderly people.

MATERIALS AND METHODS

Study population

The Rotterdam Study is an ongoing prospective population-based cohort that investigates occurrence, determinants, and consequences of diseases in an ageing population.[24] The first baseline measurements of Rotterdam Study started in 1990. After two expansions in 2000 and 2006, it comprised 14,926 participants aged 45 years and over by the end of 2008. Follow-up visits were held every 3-5 years. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus

MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport. The Rotterdam Study has been entered into the Netherlands National Trial Register (NNTR) and into the WHO International Clinical Trials Registry Platform (ICTRP) under shared catalogue number (NTR6831). All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

The present study included participants from the baseline examination of the third visit of the first cohort (RS-I-3, n=1,682), first visit of the second cohort (RS-II-1, n=1,881), the first visit of the third cohort (RSIII-1, n=3,112) and their consecutive follow-ups (RSI-4, RSII-2 and RSIII-2) (**Figure 1**).

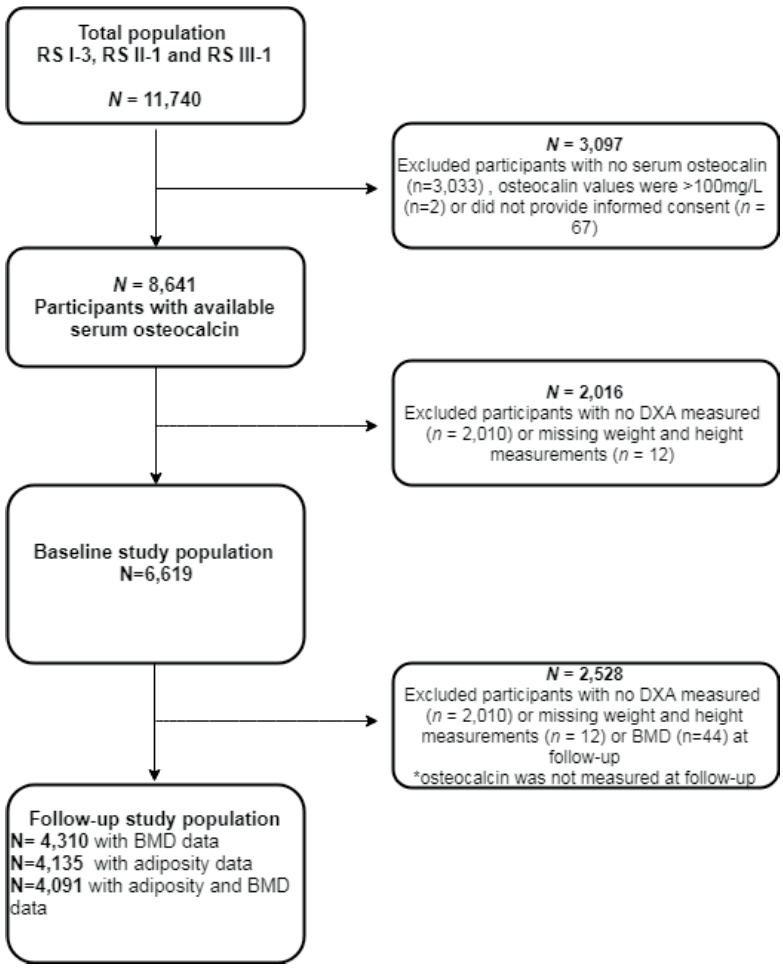


Figure 1 | Flow chart of the study population

Measurement of Total Serum Osteocalcin Levels

Total serum osteocalcin levels were measured at baseline by means of a N-MID osteocalcin electrochemiluminescence immunoassay (ELICA) kit (Roche Diagnostics, Mannheim, Germany). The monoclonal antibodies used in the test react specifically with epitopes on the N-MID-fragments and the N-terminal fragment, and detect the stable N-MID-fragment as well as the intact osteocalcin. The intact osteocalcin (amino acids 1-49) is unstable due to protease cleavage between amino acids 43 and 44. Therefore, the N-MID-fragment (amino acids 1-43) is considered more stable[25] and have been used as measure of total osteocalcin in clinical practice.

Assessment of Bone Mineral Density

Femoral neck BMD (FN-BMD) was measured by a Dual-energy X-ray absorptiometry (DXA) scanner. For RSIII-1 we used a Prodigy scanner (GE-Lunar Corp, Madison, WI, USA), and a DPX-L scanner (GE-Lunar Corp, Madison, WI, USA) was used for RSI-3 and RSII-1. In addition, we measured FN-BMD at the follow-up visits using a Prodigy scanner for RSI-4 and RSII-2 while an iDXA scanner for RSIII-2 was used. *Incidence fractures* were reported either by general practitioners (GPs) in the research area using digitalized medical records or through hospital discharge records. All events were verified by two trained research physicians which coded the events independently. Follow-up time was calculated as the time from baseline to first fracture, death or end of follow-up period (or loss to follow-up), whichever occurred first.[26]

Assessment of Adiposity

We derived several indexes of adiposity including BMI, waist circumference (WC), waist-to-hip ratio (WHR), total body fat mass and total body fat percentage. We also assessed gynoid and android fat mass as indices of fat mass distribution.

Height and body weight were measured at baseline with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). WC was measured using a tape measure at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets. Hip circumference was recorded as the maximum circumference over the buttocks. WHR was calculated as the ratio of the circumference of the waist over that of the hips. Body composition was measured by a DXA scanner (described above), in average five years after osteocalcin was measured. Fat mass of the total body and specific regions were analysed using enCORE software; deriving android and gynoid fat mass. Fat mass percentage (%) was calculated (i.e., fat mass as a percentage of total body weight).

Covariates

Information on medication use was derived from baseline questionnaires and included the use of vitamin supplementations, corticosteroids, drugs for bone diseases and antithrombotic drugs. Serum vitamin D and serum calcium were measured in blood samples using standard methods. Glucose was determined by automated enzymatic procedures in a fasting blood sample.[27] Type 2 diabetes (T2D) was defined as fasting plasma glucose >= 7mmol/l. The estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation in mL/min/1.732.[28] Total weekly duration of *physical activity* was assessed by an adapted version of the Zutphen Physical Activity Questionnaire[29] in the first and second cohort, and the LASA Physical Activity Questionnaire[30] in the third cohort. Metabolic equivalent of task (MET) values were assigned to all activities in the questionnaires and total physical activity per week was calculated.[31] Given the different type of questionnaires used, all the MET values were standardised prior analysis.

STATISTICAL ANALYSES

Phenotype Considerations

Prior analyses, we defined individuals with osteocalcin levels above 100 ng/mL as potential outliers and excluded them from the analyses (n=4). Next, given total serum osteocalcin did not followed approximately normal distribution we transformed it using an inverse normal transformation in order to model osteocalcin with a normal distribution.

Main Analyses

In the descriptive table of our study population we reported continuous variables as mean ± SD unless stated otherwise, and categorical variables were presented as percentages. We used linear regression models to examine if total serum osteocalcin levels were associated with BMD and different indices of adiposity measured at baseline and follow-up. We considered three models 1) *basic model* which was adjusted for sex, age and cohort effect; 2) *confounder model* which was additionally adjusted for physical activity, T2D, serum vitamin D, serum calcium, glomerular filtration rate, vitamin supplements, use of corticosteroid drugs, use of antithrombotic drugs and use of drugs for bone diseases and 3) *mediator model* which was adjusted additionally for BMI. Next, we preformed time to event analyses to assess the hazard risk of sustaining hip, wrist, or any non-vertebral fracture, and tested the all underlying assumptions. In order to observe the joint effect of osteocalcin and BMI on BMD we created sex and age specific tertiles of BMI and osteocalcin, resulting in 9 groups, where we compared the

prevalence of osteopenia and osteoporosis across the groups. Differences between the groups were tested using chi-square statistic. Finally, we preformed mediation analysis to test how much of the osteocalcin and BMD association is attributed to BMI using the *mediation* package in R.[32] We followed the same principle to estimate how much of the effect of osteocalcin on wrist fractures is attributed to BMD. All analyses were conducted using R (Version 3.5.1) .[33]

Sensitivity Analyses

We examined whether the association between osteocalcin and BMD or BMI differed by sex and age at the time osteocalcin was measured. Therefore, we evaluated the statistical interaction by adding the product term of osteocalcin with age or sex. If there were significant interactions ($p<0.1$), the analyses were stratified accordingly. Next, for our main analyses we only had DXA-derived estimates of adiposity measured at follow-up. Nevertheless, for a subset of our population (N=1,962) we had measurements at both follow-up and baseline. Thus, we evaluated the association between osteocalcin and DXA-derived estimates of adiposity at baseline and follow-up in this subgroup. In addition, we estimated the correlations between the adiposity measures at baseline and follow-up. Finally, we re-analysed the mediation analysis with the mediator and outcome measured five years after the exposure to avoid possible bias due to reverse causation.

Missing values were less than 10%. To adjust for potential bias associated with missing data among confounders we used multiple imputation procedure (N=10 imputations with 50 iterations). Results after the multiple imputation procedure did not differ from the original data (data not shown).

RESULTS

Characteristics of the Study Population

Our study included 6,619 participants with mean age of 62.6 ±9.1 years from which 44.2% were men. Men were taller, heavier and with higher WC compared to women. On the other hand, women had higher total body fat mass and body fat percentage compared to men. BMD was lower in women that also presented with higher total serum osteocalcin levels compared to men. Finally, during a median follow-up of 7.9 years (inter-quartile range [IQR] 5.0-12.0 years) 14% of the participants experienced at least one fracture from which the majority occurred in women (**Table 1**).

Table 1 | Descriptive statistics of the study population.

Characteristics	Total (n=6,619)	Female (n=3,693)	Male (n=2,926)
Baseline			
Age, years	62.6±9.1	62.8±9.3	62.5±8.9
Height, cm^2	168.9±9.5	163.0± 6.4	176.5±7.1
Weight, kg	78.2±14.5	72.8±13.2	84.9±13.2
BMI, kg/m^2	27.3±4.2	27.4±4.6	27.2±3.6
Waist circumference (cm)	93.6±12.1	89.6±11.9	98.6±10.3
Waist-to-hip ratio	0.89±0.09	0.85±0.08	0.95±0.08
FN-BMD, g/cm^2	0.93±0.15	0.89±0.14	0.97±0.14
Osteocalcin, ng/mL	20.3(2.7-99.2)	22.3(4.9-94.3)	19.6(2.7-99.2)
CKD-EPI, $ml/min/1.73m^2$	81.83 ±15.76	81.51±15.80	82.23±15.71
Type 2 diabetes, n (%)	589 (8.9)	274 (7.4)	315 (10.8)
Serum vitamin D, nmol/L Calcium	58.23±27.77	56.06±27.36	60.97±28.04
Serum calcium, mmol/L	2.43±0.10	2.45 ±0.10	2.42 ±0.10
Physical activity,	0.03±0.97	0.21 ±0.95	-0.19 ±0.94
Vitamins, n (%)	1347 (20.4)	864 (23.4)	483 (16.5)
Corticosteroid drugs, n (%)	69 (1.0)	38 (1.0)	31 (1.1)
Bone drugs, n (%)	95 (1.4)	85 (2.3)	10 (0.3)
Antithrombotic drugs, n (%)	1081 (16.3)	455 (12.3)	626 (21.4)
Incident fractures			
Hip fractures, n (%)	179 (2.7)	123 (3.3)	53 (1.8)
Wrist fractures, n (%)	211 (3.2)	180 (4.8)	31 (1.1)
Any-type of fracture, n (%)	933 (14.0)	644 (14.6)	269 (9.1)
Follow-up			
Age, years	66.6±7.9	66.5±8.0	66.3±7.7
Height, cm^2	169.13±9.45	163.31±6.5	176.89±7.0
Weight, kg	78.77±14.5	73.11 ±12.9	86.31±13.0
BMI, kg/m2	27.46±4.2	27.4±4.5	27.6±3.6
Waist circumference (cm)	93.43±12.3	89.0±11.6	99.3±10.56
Waist-to-hip ratio	0.90±0.09	0.85±0.08	0.97 ±0.07
Total body fat mass. kg	27.76±9.0	29.14±9.2	25.9±8.23
Body fat percentage, %	0.35±0.08	39.0±10.0	29.0±6.0
Gynoid fat mass, kg	4.14±1.4	4.65±1.4	3.46±1.1
Android fat mass, kg	2.63±1.1	2.51±1.1	2.79±1.1
FN-BMD, g/cm^2	0.91±0.1	0.87±0.1	0.96±0.1

CKD-EPI= Estimated glomerular filtration rate. Footnote: At the second round there were in total 4,135 individuals with available anthropometric and body composition data and 4,320 with FN-BMD. Round 2 was done in average five years after round 1.

Osteocalcin Correlations with Age, Weight and BMD

Serum osteocalcin levels correlated positively with age in women but not in men (**Supplementary Figure 1A**). In addition, osteocalcin was negatively correlated with weight and BMD in both men and women (**Supplementary Figure 1B-C**).

Osteocalcin Association with Bone Health

Osteocalcin was inversely associated with FN-BMD (β: -0.026, 95%CI: -0.029 to -0.022) at baseline and at follow-up (β: -0.029, 95%CI: -0.033 to -0.025) (**Table 2**). Furthermore, osteocalcin was associated with increased risk of wrist fractures (HR: 1.34, 95%CI: 1.15 to 1.55 but not with hip or all non-vertebral fractures (**Table 3**). There were negligible differences between the basic and the confounder model , suggesting that the reported associations were not driven by the confounders. The results are based on the sex-combined analyses.

Table 2 | Association of osteocalcin with FN-BMD as dependent variable measured at baseline and follow-up overall and stratified by cohort.

Baseline	**Overall (n=6,619)**	**RSI-3 (n=1,682)**	**RSII-1 (n=1,881)**	**RSIII-1 (n=3,112)**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Basic model	-0.033 (-0.036, -0.030)	-0.028 (-0.035, -0.022)	-0.031 (-0.038, -0.025)	-0.035 (-0.040, -0.031)
Confounder model	-0.036 (-0.039, -0.033)	-0.030 (-0.037, -0.024)	-0.033 (-0.040, -0.027)	-0.040 (-0.045, -0.036)
Mediator model	-0.029 (-0.032, -0.026)	-0.024 (-0.031, -0.018)	-0.025 (-0.031, -0.018)	-0.033 (-0.038, -0.029)
Follow-up	**Overall (n=4,310)**	**RSI-3 (n=1,162)**	**RSII-1 (n=773)**	**RSIII-1 (n=2,385)**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Basic model	-0.032 (-0.036, -0.028)	-0.028 (-0.035, -0.021)	-0.030 (-0.039, -0.021)	-0.035 (-0.040, -0.029)
Confounder model	-0.035 (-0.039, -0.031)	-0.031 (-0.038, -0.023)	-0.032 (-0.041, -0.022)	-0.038 (-0.044, -0.033)
Mediator model	-0.029 (-0.033, -0.025)	-0.025 (-0.032, -0.017)	-0.023 (-0.033, -0.014)	-0.031 (-0.037, -0.026)

The basic model was adjusted for age, sex, and cohort effect (for the overall analysis); **The confounder model** was additionally adjusted for physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases. **The mediator model** was additionally adjusted for BMI. β=effect estimate.

Table 3 | Association of osteocalcin with incident non-vertebral, hip or wrist fracture as outcome.

	Any-type of fracture	Hip fracture	Wrist fracture
	(935 cases/ 5,740 controls)	(179 cases / 6,496 controls)	(212 cases / 6,462 controls)
	HR (95%CI)	HR (95%CI)	HR (95%CI)
Basic model	1.01 (0.94-1.07)	0.96 (0.83-1.11)	1.30 (1.13-1.50)
Confounder model	1.04 (0.97-1.12)	0.98 (0.84-1.14)	1.34 (1.15-1.55)

The basic model was adjusted for age, sex and cohort effect. **The confounder model** was additionally adjusted for physical activity., BMI, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, and use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Osteocalcin Association with Adiposity

Inverse associations were also observed between osteocalcin and both BMI (β: -0.919, 95%CI: -1.093 to -0.745) and WC (β: -2.323, 95%CI: -2.778 to -1.867) at baseline, and with follow-up measurement of BMI (β: -0.815, 95%CI: -0.997 to -0.634) and WC (β: -2.213, 95%CI: -2.676 to -1.750) (**Table 4**). Further, osteocalcin was inversely associated with total body fat mass (β: -1.1345, 95%CI: -1.715 to -0.975), body fat

Table 4 | Association of osteocalcin with different measurements of adiposity as dependent variable. Using anthropometric measurements of adiposity at the time osteocalcin was measured (1st round) and anthropometric measurements and DXA-derived parameters of adiposity five years after osteocalcin was measured (2nd round; n=4,135).

Adiposity Outcomes:	**Women (n=2,364)**		**Men (n=1,771)**	
Baseline	β	95%CI	β	95%CI
BMI (kg/m2)	-1.016	(-1.193, -0.838)	-0.735	(-0.922, -0.549)
Waist circumference (cm)	-2.493	(-2.959, -2.027)	-1.757	(-2.297, -1.217)
Waist-to-hip ratio	-0.013	(-0.016, -0.010)	-0.013	(-0.015, -0.008)
Follow-up				
BMI (kg/m2)	-0.915	(-1.099, -0.728)	-0.765	(-0.964, -0.566)
Waist circumference (cm)	-2.402	(-2.877, -1.927)	-1.943	(-2.531, -1.355)
Waist-to-hip ratio	-0.014	(-0.017, -0.011)	-0.013	(-0.017, -0.010)
Total body fat mass, kg	-1.499	(-1.877, -1.120)	-1.441	(-1.895, -0.993)
Body fat percentage, %	-0.010	(-0.013, -0.007)	-0.011	(-0.014, -0.008)
Gynoid fat mass, kg	-0.163	(-0.223, -0103)	-0.142	(-0.203, -0.081)
Android fat mass, kg	-0.196	(-0.239, -0.152)	-0.207	(-0.266, -0.147)

The models are adjusted for age, sex, cohort effect physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases. All analyses were performed in the individuals with available data at both baseline and follow-up (n=4,135). β=effect estimate. **Footnote:** for comparison purposes the analyses at baseline and follow-up were performed in the same group of individuals. Osteocalcin was measured only at baseline.

percentage (β: -0.009, 95%CI: -0.012 to -0.007), gynoid (β: -0.140, 95%CI: -0.119 to -0.081) and android (β: -0.179, 95%CI: -0.221 to -0.137) fat mass measured at follow-up (**Table 4**). The associations attenuated after adjustment for T2D but still remained significant. The results are based on the sex-combined analyses.

Joint Effect of Osteocalcin and BMI on Bone Health

Participants with low BMI and high osteocalcin levels had significantly higher prevalence of osteopenia and osteoporosis (60.2% and 12.4%) compared to individuals with BMI and osteocalcin levels (OC) in the middle range (46.5% and 4.1%). The prevalence of osteoporosis in the highest tertile of osteocalcin levels decreased significantly with the increase of BMI (12.4% [high OC-low BMI], 5.9% [high OC-middle BMI] and 3.1% [high OC-high BMI]). In the group of participants with low OC-high BMI the prevalence of osteoporosis was the lowest (1.2%) (**Figure 2**).

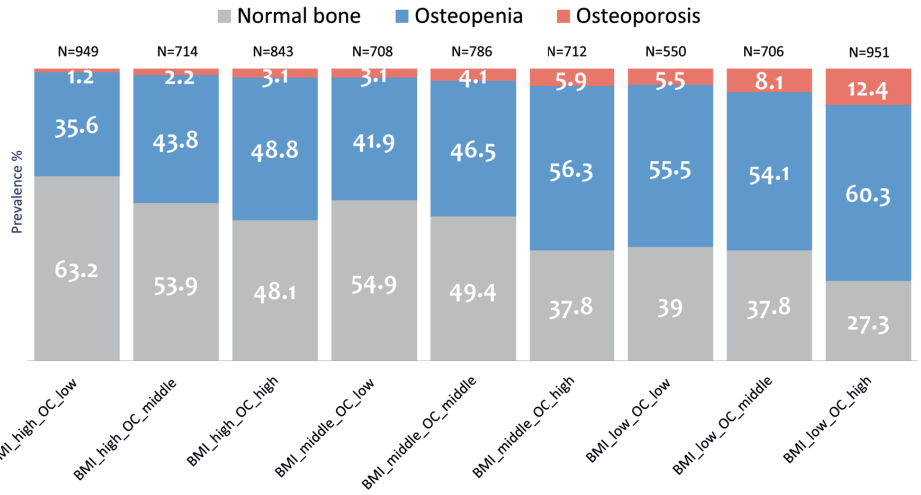


Figure 2 | Shows the prevalence of osteopenia (blue) and osteoporosis (red) according to BMI and OC sex and age adjusted tertiles

Mediation Analysis

The mediation analyses showed that significant proportion of the associations between osteocalcin and BMD was mediated by BMI. All three conditions for the mediation analysis were fulfilled. First, as we presented above, osteocalcin was significantly associated with FN-BMD and wrist fracture (Condition 1 fulfilled). Second, osteocalcin was associated with BMI (Condition 2 fulfilled). Third, BMI was associated with FN-BMD (Condition 3 fulfilled) (**Supplementary Table 1**). We showed that the total effect of osteocalcin on FN-BMD is -0.033 (95%CI: -0.036 to -0.030) while the direct effect

controlling for BMI is -0.026 (95%CI: -0.029 to -0.0022); meaning that around 23% of the association between osteocalcin and FN-BMD was mediated through BMI (**Figure 3a**). Next, we tested also how much of the association between osteocalcin and wrist fracture was medicated by BMD. We observed that only 25% of the effect of osteocalcin on wrist fractures is medicated by BMD. (**Figure 3b**).

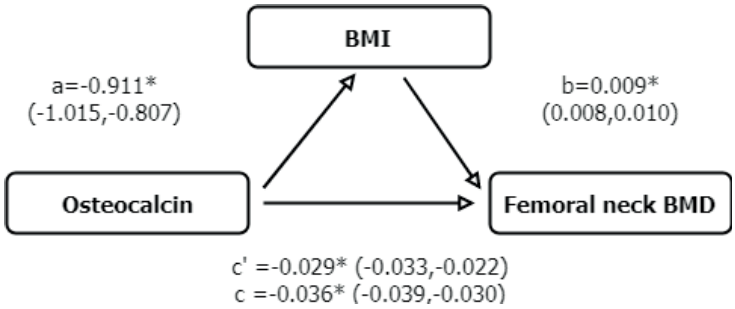


Figure 3a | Mediation analysis on the relationship between osteocalcin and femoral neck BMD. The total effect of osteocalcin on femoral neck BMD is -0.036 (path c). This effect can be mediated by BMI. The effect of osteocalcin on BMI is -0.11 (path a); the effect of BMI on femoral neck BMD, controlling for osteocalcin is 0.009 (path b); The direct effect of osteocalcin on femoral neck BMD, controlling for BMI, is -0.029 (path c'); The mediating effect of osteocalcin on femoral neck BMD through BMI is -0.007 (23%) (path a*b). Total sample size for 6,619 (baseline). * indicates significant association.

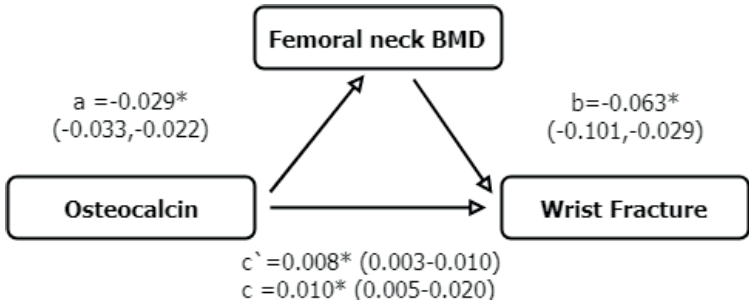


Figure 3b | Mediation analysis on the relationship between osteocalcin and wrist fracture. The total effect of osteocalcin on Wrist fractures is 0.010 (path c). This effect can be mediated by femoral neck BMD. The effect of osteocalcin on femoral neck BMD is -0.029 (path a); the effect of femoral neck BMD on wrist fracture, controlling for osteocalcin is -0.063 (path b); The direct effect of osteocalcin on Wrist fracture, controlling for femoral neck BMD, is -0.008 (path c'); The mediating effect of osteocalcin on Wrist fracture through femoral neck BMD is 0.002 (20%) (path a*b). Total sample size for 6,619 (baseline). * indicates significant association

Sensitivity Analyses

There was significant interaction (P<0.05) between osteocalcin levels and sex for the BMD and BMI analyses. Although, the association with BMD was stronger in women

compared to men (-0.030 [95%CI: -0.034, -0.026] and -0.025 [95%CI: -0.031, -0.020]) the estimates were comparable (**Supplementary Table 2**). On the other hand, the sex differences were more pronounced for the adiposity outcomes, thus, the main results were presented stratified by sex (**Figure 4**). We also observed significant interaction between osteocalcin levels and age ($p_{interaction}$= <0.01) for both BMI and BMD. After stratifying our analyses for age, we observed similar associations between osteocalcin and BMD for all age categories, however, the effect estimates were stronger among the eldest individuals (**Supplementary Table 3**). On the other hand, in elderly people, the effect of osteocalcin on BMI was less strong compared to the younger individuals (**Supplementary Table 4**). Next, in the sub-sample (n=1,962) where body composition was measured twice (baseline and follow-up visit) the association between osteocalcin and the different measures of adiposity was significant at both visits with a slight attenuation overtime for the majority of the measures (**Supplementary Table 5**). There was high correlation between the adiposity measures at both visits (**Supplementary Figure 2**). Finally, as a sensitivity step, we reanalysed the mediation analyses with the mediator and outcome measured five years after the exposure and we observed similar findings to the reported in the main analyses (**Supplementary Table 6**). Moreover, all measures of adiposity also showed significant mediating proportion which was less strong than BMI (**Supplementary Table 6**).

DISCUSSION

In this large population-based study (N=6,619), we found strong inverse associations between osteocalcin, BMD, and several distinct measures of adiposity such as BMI and fat mass. While higher serum osteocalcin levels were associated with increased risk of wrist fracture, there was no association of osteocalcin with hip or all non-vertebral fractures. We also showed that the effect of osteocalcin on BMD was partly explained by BMI (23%). Finally, individuals with high serum osteocalcin levels and low BMI may have significant deterioration of bone mass corroborated by the high prevalence of osteopenia or osteoporosis in this group.

To our knowledge, this is the first study to evaluate the joint effects of osteocalcin and adiposity on bone health. First, we found osteocalcin and BMI to be independently associated with BMD what is in line with previous reports.[8,9,13,16] Next, we observed that the prevalence of osteopenia and osteoporosis was highest in the group of people with low BMI and high serum osteocalcin levels and lowest in the opposite case (i.e., high BMI and low osteocalcin). The BMI and osteocalcin tertiles were adjusted for age and sex, thus, the differences in prevalence were not influenced by these variables. Weight dependent-loading of the bone is pivotal for the accrual

and maintaining of bone mass and strength throughout the life.[34] Therefore, decrease in weight (i.e. low BMI) leads to a reduced mechanical stress on the bone which may have detrimental effects on BMD. This is supported by our findings of high prevalence of osteoporosis in the lowest BMI group. Interestingly, the prevalence of osteoporosis in the low BMI group increased with the increase of serum osteocalcin levels. This, can simply be result of the increased bone-turnover. However, it has been postulated that osteocalcin may also act directly on the bone by inhibiting bone mineralization or indirectly by regulating global energy metabolism. Whether osteocalcin has a direct effect on bone mineral density and leads to osteoporosis or the high levels in osteoporotic individuals are only result of increased bone-turnover remains unclear. Nevertheless, the presence of low BMI and high osteocalcin levels may be an indication of deteriorated bone health.

Fractures are the most clinically relevant endpoint of osteoporosis. Therefore, it is of interest to identify other potential predictors of fracture risk beside BMD. Previously, osteocalcin has been shown to be a good predictor of hip fractures in elderly women.[11,35] This is in contrast with our findings, where we observed no effect of osteocalcin on hip fractures in both men and women. Several factors can explain these discrepancies. First, the study by Szulc et al,[35] was performed in elderly institutionalized women with relative small sample size, thus the results cannot be directly compared. Second, our population was on average 10 years younger in comparison with the other studies. As hip fractures are most common in the elderly (75+), in our population they are yet to occur. Third, there was a big difference in the follow-up time with previous studies having an average follow-up of 2-3 years while ours was longer (8.9 years) with a maximum of 15 years of follow-up. This raises the possibility that osteocalcin is only a good predictor of short-term fractures but not long-term ones. Finally, Vergnaud et al[11] found that only undercarboxylated osteocalcin but not total osteocalcin is associated with hip fractures, which is in line with our findings using total osteocalcin. Noteworthy, we did observe an association between total serum osteocalcin levels and wrist fractures. To the extent of our knowledge, this is the first study to evaluate the association between osteocalcin and wrist fractures. We observed a 20% increase in wrist fracture risk per unit increase in total serum osteocalcin in both men and women. Using mediation analysis, we showed that only 20% of the effect of osteocalcin on wrist fracture can be attributed to BMD while the remaining 80% can be results of a direct effect of osteocalcin or other pathways, not included in the analysis, on wrist fracture. The underlying mechanisms for this relationship are still unknown, but may be through both impaired bone formation[36] and bone resorption.[37] Recently, it has been postulated that osteocalcin may also enhance bone toughness by acting as bridge between the matrix and mineral fractions.[38] Thus, the increased bone-turnover coupled with impaired γ-carboxylation of osteocalcin in

elderly people can lead to abnormal bone mineralization which may affect the material properties of the bone and increase the risk of fracture. As the wrists are less mechanically loaded compared to the hip, they are more fragile and likely to fracture earlier in the life course, as shown in our previous effort.[26] However, these findings need to be validated by other large-scale studies, followed by studies to elucidate the underlying causal pathways.

As aforementioned, numerous observational studies have shown the negative association between osteocalcin levels and BMD.[8,9,39,40] Notably, most of the past research have been done in post-menopausal women whereas data on men have traditionally been scarce. Here, we confirmed the findings in women and increase the evidence in men. Moreover, we observed an increase in the effect size of 10-20% in elderly people which is in line with current evidence.[41] The age dependent effect of osteocalcin on BMD can be result of the increased bone remodelling in the eldest population leading to increase in total serum osteocalcin levels[42]. Also, it has been shown that the vitamin K-dependent carboxylation of osteocalcin to reduce with age; indicating higher levels of undercarboxylated osteocalcin in the circulation, what may influence the bone metabolism in return.

Besides being associated with BMD, osteocalcin was also negatively associated with BMI, WC and different measures of fat mass; in line with other observational studies.[13,16,43,44] The increase in fat mass was initially observed in osteocalcin deficient mice, which highlighted the endocrine role of the bone, in particular, its extra-skeletal effects.[45] Large body of evidence have implicated osteocalcin in glucose, fat and overall energy metabolism.[46,47,48] It has been shown that infusion of osteocalcin in wild mice reduced obesity,[49] what has been corroborated in human studies showing that increases in undercarboxylated osteocalcin are associated with decreases in body weight and fat.[50] On the other hand, the excess of fat mass exhibit dual and opposite effects on bone metabolism which will depend on the type of fat mass accumulation and the circulating factors; this can affect osteocalcin levels in both directions. Recently, it has been also shown that the adipose tissue may produce osteocalcin on its own.[51] This just adds up to the molecular complexity of the bone-fat interaction. Unfortunately, we were not able to test the potential bidirectional effects between osteocalcin and fat mass in our population as we did not have follow-up data on osteocalcin. Finally, we showed that around 23% of the effect of osteocalcin on BMD is mediated by BMI.

A strength of this study is the large population-based settings involving 6,619 individuals, enhancing the extrapolation of the findings to other older adults. While the vast majority of previous research have been focused largely on post-menopausal women our study included both men and women. Thus, significantly contributing to the literature regarding the effects of osteocalcin on BMD and fracture risk in men.

Moreover, this is the first study to investigate the association of osteocalcin with wrist fractures and until now the study with the longest follow-up of up to 15 years (7.9 years on average) to evaluate hip, wrist and all non-vertebral fracture risk. However, several limitations of our study need to be noted. First of all, the study was performed using a cross-sectional design with only one measurement of total serum osteocalcin. Therefore, we were not able to evaluate whether changes in osteocalcin levels have an impact on bone and fat mass changes, neither we were able to test for possible bidirectional effects and discard the possibility of reverse association. Although we had two measurements of BMD, we could not properly evaluate the effect of osteocalcin on BMD change, as a result of the software incompatibility between the first and follow-up DXA measurement. Next, the DXA derived body composition parameters were assessed five years after the measurement of osteocalcin. Total osteocalcin levels and body composition may have changed over this time period. However, there was no major variation of BMI and/or body composition among our population during this time period. We observed a strong correlation ($r>0.90$) between the anthropometric measurements of adiposity (BMI and WC) at the time osteocalcin was measured and five years after. Similar correlations were observed for the body composition parameters in a subset of individuals (N=1,962) with two available DXA measurements. In the same group, we also observed that the effect of osteocalcin on fat mass measurements was stable across visits. Therefore, if body composition was measured at the same time osteocalcin was measured, we would have observed similar findings as with the follow-up assessment. Although we made attempt to adjust for all possible confounders, we did not have available data on vitamin K supplementation or dietary vitamin K intake; which can significantly influence the levels of serum undercarboxylated osteocalcin levels. We were only able to account for overall vitamin supplementation. Next, we only measured total serum osteocalcin, while undercarboxylated osteocalcin or carboxylated osteocalcin were not measured. Animal studies have shown that undercarboxylated osteocalcin is the active form of osteocalcin;[45,49] however, these findings should be validated in humans as studies have provided conflicting results.[52] Finally, our findings can't be generalized to younger populations and or different ethnicities.

In conclusion, high osteocalcin levels and low BMI appear to play an important role in the decrease of bone mineral density, consequently leading to developing of osteoporosis and increased fracture.

REFERENCES

1 Delmas PD, Eastell R, Garnero P, Seibel MJ, Stepan J, Committee of Scientific Advisors of the International Osteoporosis Foundation]. The use of biochemical markers of bone turnover in osteoporosis. Committee of Scientific Advisors of the International Osteoporosis Foundation. *Osteoporos Int* 2000; **11 Suppl 6**: S2-17.

2 Lee AJ, Hodges S, Eastell R. Measurement of Osteocalcin. *Ann Clin Biochem An Int J Biochem Lab Med* 2000; **37**: 432–46.

3 Zanatta LCB, Boguszewski CL, Borba VZC, Kulak CAM. Osteocalcin, energy and glucose metabolism. *Arq Bras Endocrinol Metabol* 2014; **58**: 444–51.

4 Yoshikawa Y, Kode A, Xu L, *et al.* Genetic evidence points to an osteocalcin-independent influence of osteoblasts on energy metabolism. *J Bone Miner Res* 2011; **26**: 2012–25.

5 Singh S, Kumar D, Lal AK. Serum Osteocalcin as a Diagnostic Biomarker for Primary Osteoporosis in Women. *J Clin Diagn Res* 2015; **9**: RC04-7.

6 Minisola S, Rosso R, Romagnoli E, *et al.* Serum osteocalcin and bone mineral density at various skeletal sites: a study performed with three different assays. *J Lab Clin Med* 1997; **129**: 422–9.

7 Jagtap VR, Ganu J V, Nagane NS. BMD and Serum Intact Osteocalcin in Postmenopausal Osteoporosis Women. *Indian J Clin Biochem* 2011; **26**: 70–3.

8 Emaus N, Nguyen ND, Almaas B, *et al.* Serum level of under-carboxylated osteocalcin and bone mineral density in early menopausal Norwegian women. *Eur J Nutr* 2013; **52**: 49–55.

9 Szulc P, Arlot M, Chapuy M-C, Duboeuf F, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J Bone Miner Res* 2009; **9**: 1591–5.

10 Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993; **91**: 1769–74.

11 Vergnaud P, Garnero P, Meunier PJ, Bréart G, Kamihagi K, Delmas PD. Undercarboxylated Osteocalcin Measured with a Specific Immunoassay Predicts Hip Fracture in Elderly Women: The EPIDOS Study [1]. *J Clin Endocrinol Metab* 1997; **82**: 719–24.

12 Chin K-Y, Ima-Nirwana S, Mohamed IN, *et al.* Serum Osteocalcin Is Significantly Related to Indices of Obesity and Lipid Profile in Malaysian Men. *Int J Med Sci* 2014; **11**: 151.

13 Hu W, Ke Y, He J, *et al.* Serum osteocalcin levels are inversely associated with plasma glucose and body mass index in healthy Chinese women. *Acta Pharmacol Sin* 2014; **35**: 1521–6.

14 Zhou M, Ma X, Li H, *et al.* Serum osteocalcin concentrations in relation to glucose and lipid metabolism in Chinese individuals. *Eur J Endocrinol* 2009; **161**: 723–9.

15 Pittas AG, Harris SS, Eliades M, Stark P, Dawson-Hughes B. Association between Serum Osteocalcin and Markers of Metabolic Phenotype. *J Clin Endocrinol Metab* 2009; **94**: 827–32.

16 Kindblom JM, Ohlsson C, Ljunggren Ö, *et al.* Plasma Osteocalcin Is Inversely Related to Fat Mass and Plasma Glucose in Elderly Swedish Men. *J Bone Miner Res* 2009; **24**: 785–91.

17 Asomaning K, Bertone-Johnson ER, Nasca PC, Hooven F, Pekow PS. The Association between Body Mass Index and Osteoporosis in Patients Referred for a Bone Mineral Density Examination. *J Women's Heal* 2006; **15**: 1028–34.

18 Johansson H, Kanis JA, Odén A, *et al.* A Meta-Analysis of the Association of Fracture Risk and Body Mass Index in Women. *J Bone Miner Res* 2014; **29**: 223–33.

19 Elefteriou F, Takeda S, Ebihara K, *et al.* Serum leptin level is a regulator of bone mass. *Proc Natl Acad Sci* 2004; **101**: 3258–63.

20 Kajimura D, Lee HW, Riley KJ, *et al.* Adiponectin Regulates Bone Mass via Opposite Central and Peripheral Mechanisms through FoxO1. *Cell Metab* 2013; **17**: 901–15.

21 Williams GA, Wang Y, Callon KE, *et al. In Vitro* and *in Vivo* Effects of Adiponectin on Bone. *Endocrinology* 2009; **150**: 3603–10.

22 Cao JJ. Effects of obesity on bone metabolism. *J Orthop Surg Res* 2011; **6**: 30.

23 Viljakainen H, Ivaska KK, Paldánius P, *et al.* Suppressed Bone Turnover in Obesity: A Link to Energy Metabolism? A Case-Control Study. *J Clin Endocrinol Metab* 2014; **99**: 2155–63.

24 Ikram MA, Brusselle GGO, Murad SD, *et al.* The Rotterdam Study: 2018 update on objectives, design and main results. *Eur J Epidemiol* 2017; **32**: 807–50.

25 Granchi D, Gómez-Barrena E, Rojewski M, *et al.* Changes of Bone Turnover Markers in Long Bone Nonunions Treated with a Regenerative Approach. *Stem Cells Int* 2017; **2017**: 3674045.

26 Trajanoska K, Schoufour JD, de Jonge EAL, *et al.* Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. *Bone* 2018; **114**: 116–24.

27 Stolk RP, Pols HAP, Lamberts SWJ, Jong PTVM d., Hofman A, Grobbee DE. Diabetes Mellitus, Impaired Glucose Tolerance, and Hyperinsulinemia in an Elderly Population The Rotterdam Study. *Am J Epidemiol* 1997; **145**: 24–32.

28 Levey AS, Stevens LA, Schmid CH, *et al.* A New Equation to Estimate Glomerular Filtration Rate. *Ann Intern Med* 2009; **150**: 604.

29 Caspersen CJ, Bloemberg BP, Saris WH, Merritt RK, Kromhout D. The prevalence of selected physical activities and their relation with coronary heart disease risk factors in elderly men: the Zutphen Study, 1985. *Am J Epidemiol* 1991; **133**: 1078–92.

30 Stel VS, Smit JH, Pluijm SM., Visser M, Deeg DJ., Lips P. Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. *J Clin Epidemiol* 2004; **57**: 252–8.

31 AINSWORTH BE, HASKELL WL, HERRMANN SD, *et al.* 2011 Compendium of Physical Activities. *Med Sci Sport Exerc* 2011; **43**: 1575–81.

32 Tingley D, Yamamoto HT, Hirose K, Keele L, Princeton KI. mediation: R Package for Causal Mediation Analysis. http://cran.r-project.org/package=mediation (accessed June 17, 2019).

33 R: a language and environment for statistical computing. https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing (accessed Aug 17, 2017).

34 Iwaniec UT, Turner RT. Influence of body weight on bone mass, architecture and turnover. *J Endocrinol* 2016; **230**: R115-30.

35 Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. *Bone* 1996; **18**: 487–8.

36 Glowacki J, Rey C, Glimcher MJ, Cox KA, Lian J. A role for osteocalcin in osteoclast differentiation. *J Cell Biochem* 1991; **45**: 292–302.

37 Ducy P, Desbois C, Boyce B, *et al.* Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; **382**: 448–52.

38 Zoch ML, Clemens TL, Riddle RC. New insights into the biology of osteocalcin. *Bone* 2016; **82**: 42–9.

39 Vs K, K P, Ramesh M, Venkatesan V. The association of serum osteocalcin with the bone mineral density in post menopausal women. *J Clin Diagn Res* 2013; **7**: 814–6.

40 Zofková I, Hill M, Palicka V. Association between serum undercarboxylated osteocalcin and bone density and/or quality in early postmenopausal women. *Nutrition*; **19**: 1001–3.

41 Liu G, Peacock M. Age-Related Changes in Serum Undercarboxylated Osteocalcin and its Relationships with Bone Density, Bone Quality, and Hip Fracture. *Calcif Tissue Int* 1998; **62**: 286–9.

42 Melton LJ, Khosla S, Atkinson EJ, O'Fallon WM, Riggs BL. Relationship of Bone Turnover to Bone Density and Fractures. *J Bone Miner Res* 1997; **12**: 1083–91.

43 Dubnov-Raz G, Ish-Shalom S, Chodick G, Rozen GS, Giladi A, Constantini NW. Osteocalcin is independently associated with body mass index in adolescent girls. *Pediatr Obes* 2012; **7**: 313–8.

44 Kord-Varkaneh H, Djafarian K, khorshidi M, Shab-Bidar S. Association between serum osteocalcin and body mass index: a systematic review and meta-analysis. *Endocrine* 2017; **58**: 24–32.

45 Moser SC, van der Eerden BCJ. Osteocalcin—A Versatile Bone-Derived Hormone. *Front Endocrinol (Lausanne)* 2019; **9**: 794.

46 Sabek OM, Nishimoto SK, Fraga D, Tejpal N, Ricordi C, Gaber AO. Osteocalcin Effect on Human β-Cells Mass and Function. *Endocrinology* 2015; **156**: 3137–46.

47 Huang L, Yang L, Luo L, Wu P, Yan S. Osteocalcin Improves Metabolic Profiles, Body Composition and Arterial Stiffening in an Induced Diabetic Rat Model. *Exp Clin Endocrinol Diabetes* 2017; **125**: 234–40.

48 Ferron M, McKee MD, Levine RL, Ducy P, Karsenty G. Intermittent injections of osteocalcin improve glucose metabolism and prevent type 2 diabetes in mice. *Bone* 2012; **50**: 568–75.

49 Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci* 2008; **105**: 5266–70.

50 Schafer AL, Sellmeyer DE, Schwartz A V, *et al.* Change in undercarboxylated osteocalcin is associated with changes in body weight, fat mass, and adiponectin: parathyroid hormone (1-84) or alendronate therapy in postmenopausal women with osteoporosis (the PaTH study). *J Clin Endocrinol Metab* 2011; **96**: E1982-9.

51 Foresta C, Strapazzon G, De Toni L, *et al.* Evidence for Osteocalcin Production by Adipose Tissue and Its Role in Human Metabolism. *J Clin Endocrinol Metab* 2010; **95**: 3502–6.

52 Shea MK, Gundberg CM, Meigs JB, *et al.* Gamma-carboxylation of osteocalcin and insulin resistance in older men and women. *Am J Clin Nutr* 2009; **90**: 1230–5.

SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Association of BMI with FN-BMD as dependent variable stratified by cohort.

	Overall (n=6,619)	RSI-3 (n=1,682)	RSII-1 (n=1,881)	RSIII-1 (n=3,112)
β	0.008	0.009	0.010	0.007
95%CI	(0.007,0.009)	(0.007,0.011)	(0.009,0.012)	(0.006,0.008)

The model is adjusted for age, sex, cohort effect, osteocalcin, physical activity and T2D

Supplementary Table 2 | Association of osteocalcin with FN-BMD as dependent variable stratified by sex.

	Women	Men
β	-0.030	-0.025
95%CI	(-0.034, -0.026)	(-0.031, -0.020)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 3 | Association of osteocalcin with FN-BMD as dependent variable stratified by age.

Age	<55 (n=1,240)	55-59 (n=1,667)	60-64 (n=1,525)	65-69 (n=767)	70-74 (n=624)	75-79 (n=476)	80-85 (n=226)	>85 (n=94)
β	-0.030	-0.034	-0.033	-0.040	-0.042	-0.036	-0.047	-0.054
95% CI	(-0.037, -0.022)	(-0.041, -0.028)	(-0.040, -0.025)	(-0.050, -0.031)	(-0.052, -0.032)	(-0.048, -0.023)	(-0.064, 0.031)	(-0.082, -0.025)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 4 | Association of osteocalcin with BMI as dependent variable stratified by age.

Age	<55 (n=1,240)	55-59 (n=1,667)	60-64 (n=1,525)	65-69 (n=767)	70-74 (n=624)	75-79 (n=476)	80-85 (n=226)	>85 (n=94)
β	-0.830	-0.913	-1.077	-0.806	-0.869	-0.787	-0.109	0.411
95% CI	(-1.107, -0.554)	(-1.126, -0.699)	(-1.288, -0.866)	(-1.090, -0.522)	(-1.163, -0.574)	(-1.105, -0.470)	(-0.603, 0.385)	(-0.436, 1.257)

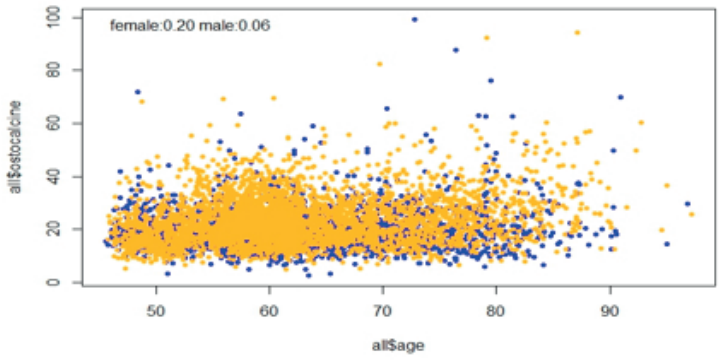
The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 5 | Association of osteocalcin with different measurements of adiposity as dependent variables in a subset of the population with measurements available at the two rounds (n=2,038).

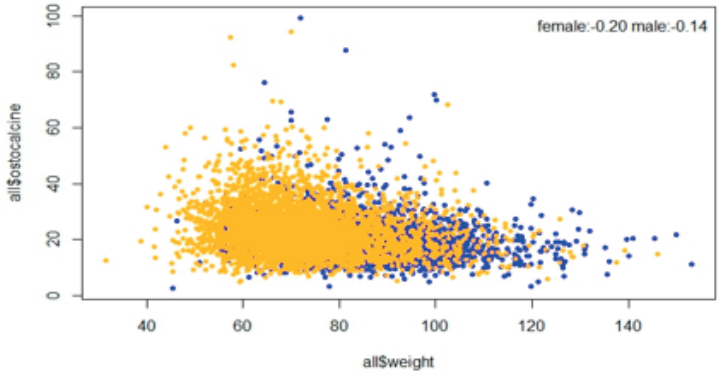
Adiposity Outcomes:	**Baseline**		**Follow-up**	
	β	95%CI	β	95%CI
Total body fat mass, kg	-1.537	(-1.949, -1.124)	-1.368	(-1.773, -0.962)
Body fat percentage,%	-0.977	(-0.014, -0.007)	-0.008	(-0.011, -0.005)
Gynoid fat mass, kg	-0.151	(-0.215, -0.087)	-0.136	(-0.197, -0.075)
Android fat mass, kg	-0.205	(-0.251, -0.159)	-0.207	(-0.258, -0.156)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases

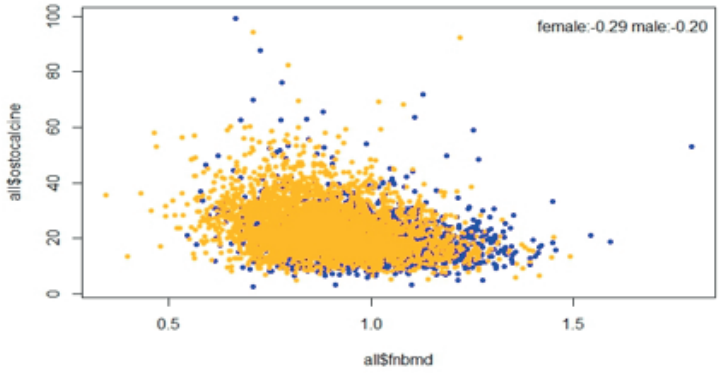
A)



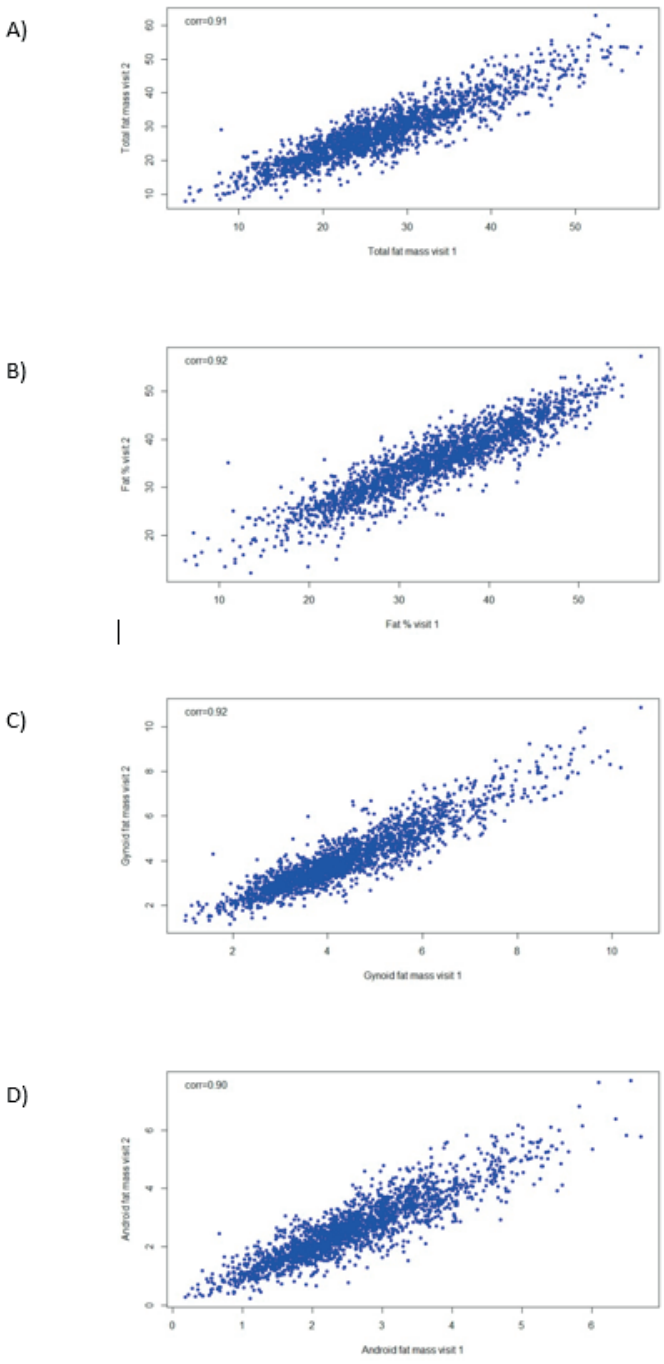
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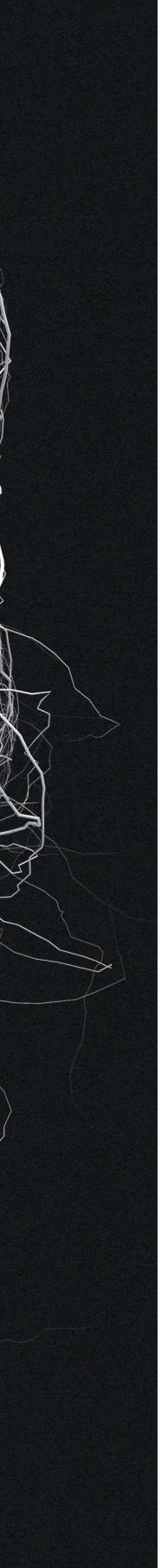


Supplementary Figure 1 | Scatter plot of osteocaline versus A) age , B) weight and C) femoral neck BMD, Female yellow ; Male blue



Supplementary Figure 2 | Scater plot of the correlation between body composition at visit 1 and visit 2 A) Total body fat mass, B) Body fat percentage, C) Gynoid fat mass and D) Android fat mass for subsample of the population where measurments at the both visit were done (n=1,962).

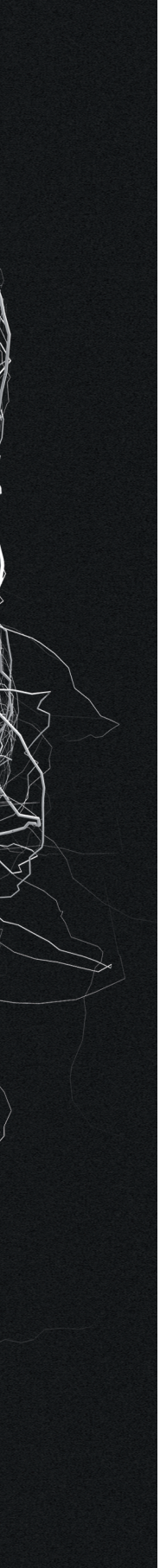




2.2

Genetic Studies





2.2.1

The genetic architecture of osteoporosis and fracture risk

Katerina Trajanoska, Fernando Rivadeneira

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ABSTRACT

Osteoporosis and fracture risk are common complex diseases, caused by an interaction of numerous disease susceptibility genes and environmental factors. With the advances in genomic technologies, large-scale genome-wide association studies (GWAS) have been performed which have broadened our understanding of the genetic architecture and biological mechanisms of complex disease. Currently, more than ~90 loci have been found associated with DXA derived bone mineral density (BMD), over ~500 loci with heel estimated BMD and several others with other less widely available bone parameters such as bone geometry, shape, and microarchitecture. Notably, several of the pathways identified by the GWAS efforts correspond to pathways that are currently targeted for the treatment of osteoporosis. Overall, tremendous progress in the field of the genetics of osteoporosis has been achieved with the discovery of *WNT16, EN1, DAAM2,* and *GPC6* among others. Assessment of the function and biological mechanisms of the remaining genes may further untangle the complex genetic landscape of osteoporosis and fracture risk. With this review we aimed to provide a general overview of the existing GWAS studies on osteoporosis traits and fracture risk.

INTRODUCTION

Osteoporosis is a progressive silent disease with devastating clinical and economic consequences. Approximately 1/3 of postmenopausal women suffer osteoporosis worldwide and at least half of these will experience a fragility fracture during their lifetime. Fragility fractures are often associated with increased morbidity and mortality, dramatically decreasing quality of life.[1-3] As population age, the prevalence of osteoporosis and its sequelae will increase substantially, becoming one of the largest global healthcare burdens.

Osteoporosis and fracture risk are determined by a complex interplay of genetic and environmental factors. Positive family history of osteoporosis is an important risk factor for fracture, which underscores the pivotal relationship between an individual's genetic makeup and disease susceptibility. Many monogenic forms of bone fragility have been identified, which are caused by a single mutation in a gene that has a major role in skeletal biology such as observed in X-linked osteoporosis, osteogenesis imperfecta and Paget disease among many others.[4] However, these monogenic mutations explain a very small fraction of the variation in bone mineral density (BMD) and osteoporosis risk in the general population. Just as in other complex diseases, advances in high-throughput genomic technologies, increasing insight on how genetic variation is organized in the genome (i.e., HapMap project) and the availability of large biobank studies have led to the advent of genome-wide association studies (GWAS) in the osteoporosis field. Given the enormous progress in the genomics of osteoporosis, the aim of this review is to provide a general overview of the existing GWAS studies on osteoporosis and fracture risk.

HERITABILITY OF BONE PROPERTIES

In order to evaluate the genetic architecture of any trait it is important first to establish if that trait is heritable. DXA derived BMD, used for the diagnosis of osteoporosis, is a highly heritable trait (h^2 =50-80%) and an excellent biomarker capturing intrinsic properties of bone biology that have led to the identification of hundreds of associated loci.[5] Twin and family studies have also shown that other bone parameters like geometry (h^2=30–70%),[6] bone ultrasound measures (h^2=40-50%)[5] and high resolution peripheral quantitative computed tomography (HR-pQCT) measures of bone micro-architecture (h^2=20–80%)[7] are also highly heritable. Such other determinants of bone strength like geometry, cortical thickness and porosity, trabecular bone morphology and intrinsic properties (quality) of bone tissue contribute to the genetic predisposition to fragility fractures but have been less well-studied due to limita-

tions in sample size. Large samples are needed since all these bone parameters are expected to be highly polygenic (i.e., determined by many variants with small effects).

FRACTURE RISK: AN EXTREMELY COMPLEX TRAIT

Numerous genetic and environmental factors, individually or combined, can cause fracture. These risk factors can act through one or multiple systems, triggering different processes across hormonal/metabolic, biomechanical and material property pathways, which eventually in combination will lead to fracture. Importantly, certain non-genetic factors can exert their influences during different life stages where distinct processes have greater influence like bone accrual in children or bone loss with aging. Genes can act alone or in combination with environmental factors (gene x environment interactions) to have an effect on fracture risk (**Figure 1**). Sometimes it may be difficult to distinguish fractures with high environmental influence from fractures having considerable influence from genetic variants. In addition, the musculoskeletal system undergoes adaptation, in principle directed to repair frequent and repetitive

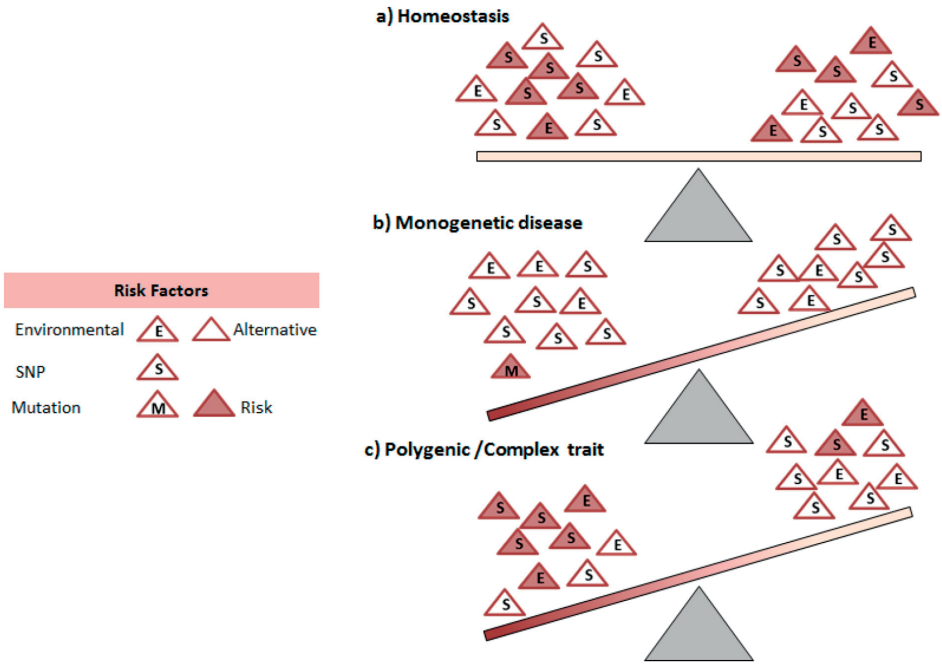


Figure 1 | Genetic and environmental factors both act on the musculoskeletal system and increase the risk of fracture. Complex traits influenced by environmental factors and/or genetic variants with weak effects need to synergize their effects in order to increase fracture risk. By contrast, monogenetic conditions are sufficient on its own to cause fracture.

micro-damage and to preserve bone-strength to the needs set by strain and stresses. This adaption can also occur in presence of genetic susceptibility to fracture, making the search for fracture genes even more difficult.

Phenotype definition is also an important determinant of the success of GWAS, frequently confronted with a trade-off between accurate trait definition and sample size. Ideally, one would choose to study fractures with a strong hereditary component i.e., low-trauma fractures occurring after falls from a standing height or less which are the consequence of osteoporosis (BMD T-score < −2.5). Nevertheless, it is well established that the majority of fractures occur above the T-Score osteoporosis threshold[8] in individuals with osteopenia (low BMD with −2.5 < T-score < −1.0). Moreover, low-trauma fractures may also occur in individuals with normal BMD (T-score > −1.0), who are for example carriers of rare mutations which affect distinct bone properties such as in the monogenic forms of hypophosphatemic rickets or osteogenesis imperfecta. These fractures occur beyond what is expected from a low BMD level and are typically attributed to impaired "bone quality" – an ambiguous term for bone properties influencing mechanical performance but not well-characterized by BMD. The mechanistic pathways affecting bone quality have been set to arise from three distinct tissue material properties that describe mechanical failure of bone: including tissue strength, fracture toughness and fatigue strength.[9] Lacking to date adequate, affordable and wide-spread methods to assess bone quality, it is no wonder that the majority (if not all) current GWAS have focused on BMD-based and fracture risk traits. Interestingly, as described below all fracture risk loci identified to GWAS are BMD loci, further supporting the mediating role of BMD on fracture. Overall, the GWAS field has prioritized sample size (high powered setting) over the use of accurate phenotype definition as the most cost-effective strategy to reduce the noise introduced by phenotypic heterogeneity and identify real genetic signals.

GENETIC STUDIES OF BONE MINERAL DENSITY

The early genetic studies in the osteoporosis field were confined to linkage and candidate gene association studies, which have been particularly focused on BMD. However, these studies have turned out to be typically underpowered leading to negative or irreproducible findings. With the advent of GWAS a new era in bone genomics has begun. The findings from the first GWA study from the Framingham study were initially underwhelming as a result of poor marker density (100,000 SNPs) and inadequate statistical power (N_{total} = 1141).[10] Thanks to the advances in genomic technologies and statistical genetic methodologies it became clear that the success of GWAS depends on sample size, effect size of causal genetic variants, the frequency

of those variants and the LD between observed genotyped variants and the unknown causal variants.[11] This knowledge has yielded a dramatic change shaping the success of subsequent GWAS, where with the rise of sample size the loci associated with BMD started to increase dramatically.

In early 2008 two GWAS simultaneously identified five common variants associated with BMD variation in the general population. Richards et al.[12] identified two variants associated with lumbar spine and femoral neck BMD mapping to *LRP5* and *TNFRSF11B (OPG*) in 8557 individuals. The *LRP5* variants were also associated with osteoporotic fractures, reproducing the findings of the largest candidate gene study drawn by the GENOMOS consortium.[13] Almost simultaneously, Styrkársdóttir et al.[14] identified in addition to *TNFRSF11B (OPG)* variants mapping to *ESR1, ZBTB40* and the major histocompatibility complex (MHC) loci in 13,786 Icelandic individuals, associated with BMD and osteoporotic fractures. Subsequent work by the same group with expanded sample size (Ntotal = 15,375) identified additional novel variants mapping to/near *TFNRSF11A (RANK), SOST, MARK3,* and *SP7* (osterix).[15] The latter gene (*SP7*) was also identified in the first BMD GWAS of children[16] (N_{total} = 5275). Medina-Gomez et al.[17] identified variants in *WNT16* associated with skull and total body BMD in children, accompanied by an effort identifying variants in the same locus associated with pQCT and wrist fracture outcomes.[18] The largest yield in discoveries has been facilitated by the rise of collaborative networks giving way to large-scale GWAS meta-analyses that identified novel bone regions and pathways. The first meta-analysis of the GEFOS consortium (N_{total} = 19,195) identified 13 novel loci associated with BMD[19] followed by a second GEFOS meta-analysis ($N_{discovery}$ = 32,961) which replicated the majority of known BMD loci and identified additional 32 novel loci (N_{total} = 83,894).[20] Fourteen of the BMD-associated loci were also associated with osteoporotic fractures, with those mapping to *FAM210A, SLC25A13, LRP5, MEPE, SPTBN1* and *DKK1* showing strongest association. This study was also the first one to identify sex-specific effects by examining the X chromosome. The variant (rs5934507), associated with BMD mapping to Xp22.31, has been previously associated with male serum testosterone levels.[21] The latter study was followed by additional large meta-analysis by Zheng et al. [22] (described below) which have provided evidence that low-frequency non-coding variants have large effects on BMD and fracture. Next, in 2018 Medina et al. [23] in a meta-analysis of 30 GWASs (N_{total} = 66,628) identified 80 loci associated with total body BMD, of which 36 had not been previously identified. Moreover, in the age-stratified analyses only two loci displayed evidence for age-specific effects, including variants in *ESR1* and in close proximity to *RANKL*. These findings suggest that most of the genes identified throughout the life-course, exert an effect on peak BMD acquisition and this effect can still be observed decades later.[23] Three recent studies have used bone mineral density estimated from heel ultrasound (eBMD), in the UK Biobank Study. In the first

effort (N_{total} = 142,487) Kemp et al. [24] identified 203 loci associated with eBMD and in the following effort (N_{total} = 426,924) this number was increased to 518 (301 novel).[25] In the latter UK biobank setting, Kim identified 613 novel loci using less stringent conditional analysis.[77] These studies highlight the value of expanding the sample size for GWAS and the amazing opportunities to unravel novel biology provided by the approach. To date >20 GWAS have been published for different bone parameters from which three are large meta-analysis and three are based on the UKBiobank study (**Table 1**). The majority of the GWA studies have scrutinized common variants (MAF > 5%). All these efforts have identified variants together ex-plaining 10–20% of the variance in bone phenotypes. It is well established that less-common (rare) variants can have bigger effects than those from common ones. An alternative approach is to focus on individuals with extremely low or high BMD in order to identify rare variants with relatively large effect. In line with this contention, whole- genome sequencing (WGS) efforts have been also successful in mapping rare variants associated with different monogenetic conditions. Using WGS and imputation to larger population sets, a rare novel variant (MAF = 0.17%) was associated with low BMD (4,931 low BMD cases and 69,034 controls) and fracture risk as a result of rare nonsense mutation within *LGR4* (c.376C > T).[36] A few years later, also using WGS and imputation the same group discovered two rare mutations in *COL1A2* associated with low BMD (2,984 cases and 206,675 contorts) in participants without signs of osteogenesis imperfecta. In 2018 Duncan et al. [27] performed the most comprehensive extreme phenotype study in 240 individuals from UK with extreme high BMD (Z-scores ≥ + 3.2) and 1955 women with high (N = 1,055) or low (N = 900) BMD. The analyses yielded two novel loci mapping near *NPR3* (rs9292469; MAF = 0.33%) associated with lumbar spine BMD and *SPON1* (rs2697825; MAF = 0.17%) associated with total hip BMD. Finally, Zheng et al.[22] using an extremely powerful WGS design identified novel rare variants associated with BMD variation in the general population. The rare noncoding variant mapped to *EN1* and showed large effects on BMD (N_{total} = 53,236, effect size = +0.20 standard deviations [SD]) and fracture risk (N_{total} = 508,253, OR = 0.85).

GENETIC STUDIES OF FRACTURE RISK

Fracture is the most important clinical outcome of osteoporosis. In the past, most of the genes shown to be associated with fracture risk have been discovered by testing known GWAS BMD loci for association with fracture as described above. To date two GWAS have been performed using vertebral fractures as an endpoint. In the first meta-analysis one locus on chromosome 16q24 (rs11645938) was associated with the risk of radiographic vertebral fractures, which failed to replicate across 5720 cases and

Table 1 | GWASs for different bone parameters and osteoporotic fractures.

Study	Trait	Sample size	Total number of GWS loci	Total number of GWS novel loci	Genes
Richards et al. (2008)	Lumbar spine and femoral neck BMD	8,557	2	2	*TNFRSF11B, LRP5*
Styrkarsdottir et al. (2008)	Lumbar spine and femoral neck BMD	13,786	4	3	*RANKL, OPG, ESR1, ZBTB40, MHC*
Styrkarsdottir et al. (2008)	Lumbar spine and femoral neck BMD	15,375	7	4	*SOST, MARK3, SP7, TNFRSF11A*
Timpson et al. (2009)	Lumbar spine and femoral neck BMD	5,275	1	1	*SP6*
Xiong et al. (2009)	Lumbar spine and femoral neck BMD	9,109	2	2	*ADAMTS18, TGFBR3*
Rivadeneira et al. (2009)	Lumbar spine and femoral neck BMD	19,195	20	13	*GPR177, SPTBN1, CTNNB1, MEPE, MEF2C, STARD3NL, FLJ42280, LRP4, ARHGAP1, F2, DCDC5, SOX6, FOXL1, HDAC5, CRHR1*
Zhao LJ et al. (2009)	Femoral neck bone geometry	5,676	1	1	*RTP3*
Guo et al. (2010)	Hip BMD and osteoporotic fractures	11,568	1	1	*ALDH7A1*
Kung et al. (2010)	Lumbar spine and femoral neck BMD	18,898	1	1	*JAG1*
Hsu et al. (2010)	Lumbar spine and femoral neck BMD	11,290	4	0	
Koller et al. (2010)	Lumbar spine and femoral neck BMD	2,193	0	0	
Paternoster L et al. (2010)	Cortical vBMD	5,789	1	0	*RANKL*
Duncan et al. (2011)	Extreme high or low hip BMD	21,798	2	2	*GALNT3, RSPO3*
Estrada et al. (2012)	Lumbar spine and femoral neck BMD	83,894	56	32	**DNM3, ANAPC1, LEKR, IDUA,WNT16, FUBP3, MPP7, MBL/DKK1, AXIN1, SOX9, FAM9B/KAL1*
Zheng et al. (2012)	Cortical bone thickness. forearm BMD and fracture	5,878	1	0	*WNT16*
Medina-Gomez et al. (2012)	Total Body BMD	13,712	1	0	*WNT16*
Paternoster L et al. (2013)	Cortical and trabecular vBMD	6,930	5	1	*RANKL, OPG, ESRR, LOC285735, FMN2/GREM2*
Zhang et al. (2013)	Lumbar spine. hip and femoral neck BMD	27,061	15	2	*SMOC1, CLDN14*

Table 1 | GWASs for different bone parameters and osteoporotic fractures. (*continued*)

Study	Trait	Sample size	Total number of GWS loci	Total number of GWS novel loci	Genes
Moayyeri A et al. (2014)	BUA. VOS. BMD	15,514	9	1	*TMEM135*
Oei et al. (2014)	Radiological vertebral fractures	2,995	1	1	16q24
Zheng et al. (2015)	Lumbar spine. femoral neck and forearm BMD	53,236	1	1	*EN1*
Pei et al. (2016)	Hip trochanter and intertrochanteric BMD	9,174	3	1	*RP11-384F7.1*
Mullin et al. (2017)	BUA and VOS	16,627	8	3	*PPP1R3B, LOC387810, SEPT5*
Kemp et al. (2017)	heel BMD	142.487	203	153	**GPC6*
Medina-Gomez et al. (2018)	Total Body BMD	66,628	80	36	**RERE, CSF1, SLC8A, PLCL1, AQP1, SMAD9, TOM1L2, ADAMTS5, ETS2*
Duncan et al. (2018)	Extreme high or low BMD	2,195	4	2	*NPR3, SPON1*
Braid et al. (2018)	(DXA)-derived hip shape	15.934	8	8	*FGFR4, ASTN2*,17q42.3, 14q32.13, 12p11.22, 21q21.1, 4p15.33, 4q31.21, 17q24.3
Kim (2018)	heel BMD	394,929	899	613	**WNT1, RSPO3, ESR, SPTBN1*
Morris et al. (2018)	heel BMD	426.824	518	301	**DAAM2*
Alonso et al. (2018)	Clinical vertebral fractures	10,683	1	1	2q13
Trajanoska et al. (2018)	Any type of fractures	562,258	15	9**	*SPTBN1, CTNNB1, RSPO3, ESR1, WNT16, STARD3NL, GRB10, FUBP3, MBL2/DKK1, LRP5, SOST, FAM210A, ETS2*

BMD = Bone mineral density, BUA broadband ultrasound attenuation, VOS = velocity of sound. DXA = Dual energy x ray absorptiometry, v = volumetric. *due to the large number of genes the functional validated genes or a smaller curated list is presented. ** Novel in relation with fracture all of the loci are known BMD loci. † closest genes.

21,791 controls.[28] A recent meta-analysis reported a locus mapping on chromosome 2q13 to be significantly associated with clinical vertebral fractures.[29] The first GWAS study on non-vertebral osteoporotic fractures (N = 700) was performed in elderly Chinese individuals and identified one fracture-associated locus within the *ALDH7A1* gene.[30] However, this gene failed to replicate in any of the larger European meta-analyses. In 2018, Trajanoska et al.[31] conducted the largest GWAS on osteoporotic fractures to date comprising 37,857 cases and 227,116 non-cases with replication in up to 300,000 individuals (147,200 cases). Altogether, the effort identified 15 fracture loci with modest effects. Interestingly, all identified loci were known BMD loci. Overall, the effect of these SNPs on fracture was smaller than the effect on BMD (**Figure 2**). Thus, the genetics of any-type of fractures in the general population is mediated through the genetic influence on BMD. This is well characterized by the genetic correlations of fracture risk with BMD. Further, among 15 tested clinical factors (including vitamin D levels and milk calcium intake) only BMD had a major causal effect on fracture.[31]

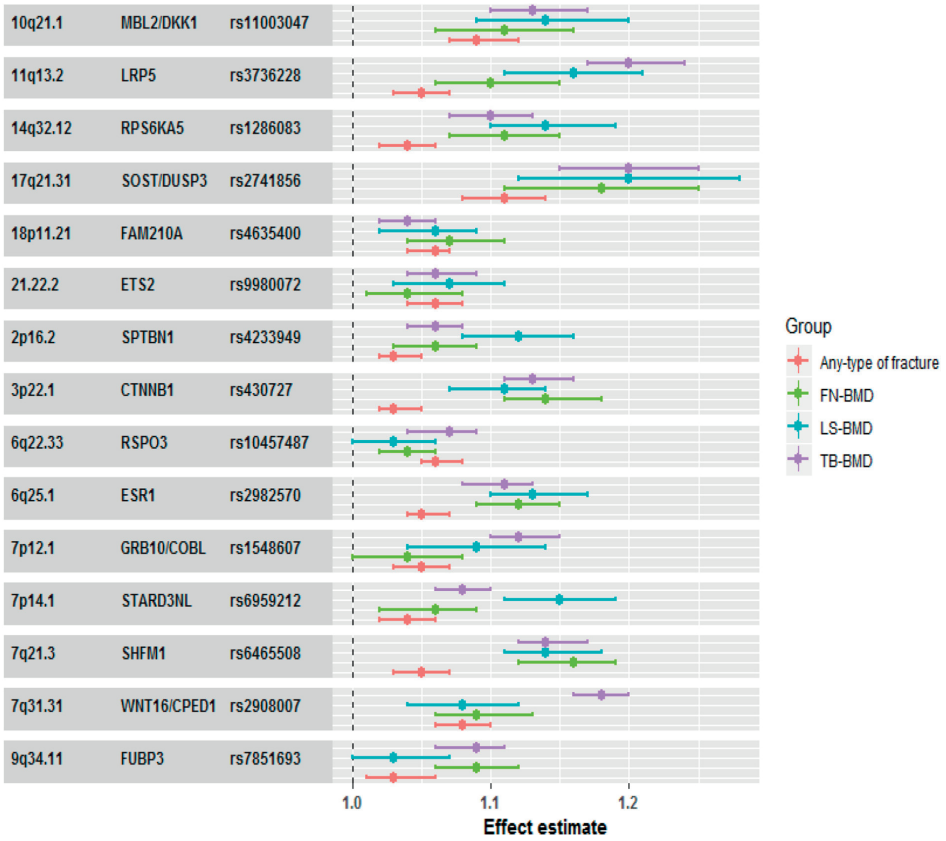


Figure 2 | Phenotype-wide effect for the fracture loci associated with femoral neck, lumbar spine and total body bone mineral density. Figure 2 was modified from Estrada et al.

GENETIC STUDIES OF OTHER BONE PARAMETERS

Studies have been performed on other bone parameters. Loci mapping near *RANK/OPG* have been associated with cortical volumetric BMD. [32,33] While genetic variants in the *FMN2/GREM2* locus were associated with trabecular volumetric BMD and fracture risk. [33] Moreover, five loci have been reported to be associated with lumbar spine volumetric BMD (Ntotal = 15,275) mapping near *WNT4* and *ZBTB40, TNFRSF11B, AKAP11*, and *TNFSF11*; from which two loci (5p13 and 1p36.12) were associated with vertebral fractures. [34] Several GWA studies have identified *RAP1A, TBC1D8*, and *OSBPL1A* to be associated with hip structure analysis (HSA) parameters. [35] Finally, Baird et al. [36] identified nine loci associated with hip shape. Seven SNPs were within 200 kb of genes involved in endochondral bone formation, namely *SOX9, PTHrP, RUNX1, NKX3-2, FGFR4, DICER1*, and *HHIP*. [36]

VARIANCE EXPLAINED IN BONE TRAITS BY BONE-ASSOCIATED VARIANTS

Harnessing the information from GWAS can help improve risk prediction of a particular disease; ultimately, this information can then be used for the prevention, diagnosis, prognosis, and treatment of a particular disease.[37] However, clinically-relevant prediction was not achieved by earlier genomic studies in the osteoporosis field; i.e., genetic risk scores did not substantially increase osteoporosis or fracture risk discrimination above the use of traditional clinical risk factors, probably as a result of low variance explained by the genetic variants. [20] The discovery of new loci using more powerful settings have led to substantial leaps in the variance explained (**Figure 3**) which will substantially improve risk prediction models in the osteoporosis field. So far, common to less-frequent genetic variants explain around 20% of the BMD variation, which is up to two-fold higher of what can be achieved through the use of other traditional risk factors for osteoporosis such as age or weight (8–9%).[38] Moreover, the combination of genetic factors, height, weight, age, and sex explains around 25% of the variance in eBMD.[77] This implies that the use of genetic markers has started to materialize in the clinical setting.

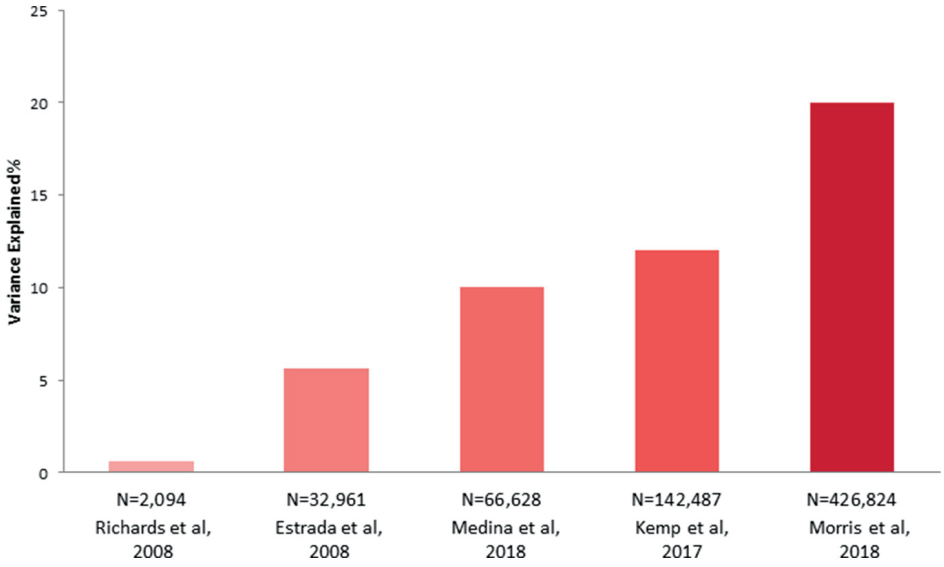


Figure 3 | Variance explained by GWAS on bone traits. With the increase of the sample size the variance explained increased accordingly.

BIOLOGICAL PATHWAYS UNDERLYING COMMON BONE CONDITIONS

Impressively, a large proportion of the genes discovered by GWAS are located in well-known bone-active pathways. Overall, there are five main pathways crucial for bone metabolism i) Mesenchymal cell differentiation ii) WNT, iii) NOTCH, iv) Hedgehog and v) OPG-RANK-RANKL signalling pathways.

Mesenchymal cell differentiation pathways

Mesenchymal stem cells (MSc) have the capacity to differentiate into several cell lineages including osteogenic and chondrogenic. Numerous transcription factors (TFs) such as RUNX2, Osterix (Osx), SOX9, can induce the osteogenic differentiation of MSc. [39-41] These factors have been also identified in various BMD GWAS. ***RUNX2*** is involved in both chondrocyte osteoblast differentiation [42] and is crucial for the early stages of osteoblast development whereas the ***Osx*** effects are more pronounced in the later stages (i.e. pre-osteoblast differentiation into functional osteoblast). [43]

WNT signalling

WNT signalling plays a pivotal role in bone development during embryogenesis and bone formation, resorption and accrual during postnatal growth. WNTs are proteins secreted from the cells and regulate the proliferation, differentiation, and apoptosis

of bone cells. [14,44] There are several pathways that can be activated by Wnt proteins among which the Wnt/β-catenin (canonical) pathway is the most important one for bone biology. This pathway is activated when WNTs proteins bind to the Frizzled membrane receptors and the low-density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6). Thereupon the stabilized β-catenin is translocated to the nucleus where it binds to the TFs LEF1/TCF and initiates the transcription of the target genes. Many genes within the *WNT* pathway have been identified by GWAS: *LRP5, WNT16, AXIN1, CTNNB1, DKK1, WLS, LPR4, MEF2C, RSPO3, SERP4, SNT16, SOST, WNT4, WNT5B*, and *EN1*. *LRP5* (encoding LDL receptor-related protein 5) is one of the first genes discovered in the osteoporosis field long before the GWAS era. [45-47] L*PR5* is a key component of the Wnt signalling since it acts as a co-receptor that binds Wnt proteins with Frizzled-receptors. Functional studies have shown that *LRP5* can lead to low bone mass (loss-of-function) [48] and high bone mass (gain-of-function mutation).[49] *LRP5* can be inhibited by several factors such as sclerostin and the dickkopf (DKK) proteins, thus, inhibit the formation of new bone. *WNT16* biology has been confirmed by many GWAS studies in relation with different bone parameters such as areal BMD, ultrasound BMD, cortical thickness and fracture, both in adults and children [17,18,20,24,31] and in peri-menopausal women.[50] Functional studies initially showed that the Wnt16−/− mouse model has reduced cortical, but not trabecular, bone mass. This effect mediated by WNT signalling, was shown to inhibit human and mouse osteoclastogenesis through direct effects on osteoclast progenitors and indirect effects resulting in increased Opg expression in osteoblasts.[51] Subsequent work showed that overexpression of WNT16 increases mainly trabecular bone mass; and postulated that *WNT16*-targeted therapies might be useful for the treatment of postmenopausal trabecular bone loss as these effects were independent of oestrogen action.[52] One of the strongest associations implicates *MEF2C* (Myocyte enhancer factor-2), which regulates a group of transcriptional regulatory proteins relevant for skeletal muscle development. Recent studies have found that a super activating form of *MEF2C* causes precocious chondrocyte hypertrophy, ossification of growth plates, and dwarfism.[53] Studies have reported that loss-of-function mutations in *WNT1* can cause dominantly inherited early-onset osteoporosis.[54] In addition, *WNT1* deletion in osteocytes has been related with low bone mass and spontaneous fractures. [55] In addition, the same study has also shown that it increases the number of osteoblasts and regulates their activity. *EN1* is another important novel gene involved in murine calvarial osteoblast differentiation and proliferation [56] and large effect on BMD and fracture risk in humans. [22]

NOTCH signalling

In the skeleton, both osteoblasts and osteoclasts require NOTCH signalling for proper differentiation and function, and the specific roles of NOTCH are dependent on the

differentiation status of the cell.[57] NOTCH is a family of four transmembrane proteins (NOTCH1–4) that are expressed on the cell surface and require cell-to-cell contact for activation [58] through several ligands (Dll1, Dll3, Dll4, JAG1, JAG2) binding to receptors expressed on the surface of neighboring cells. The ligand binding induces proteolytic cleavage and releases the NOTCH intracellular domain (NICD), which enters the cell nucleus and the transcription of NOTCH targeted genes starts. There is also crosstalk between the NOTCH and WNT signalling pathways which precise mechanisms are still not clear. Several BMD genes have been related to this pathway such as *JAG1, MAPT*, and *NOTCH2*.

Indian Hedgehog (IHH) signalling

The IHH signalling pathway consists of essential signalling molecules crucial for intra-membranous ossification of cranial bones and endochondral ossification in other parts of the skeletal system. During endochondral ossification, chondrocytes differentiate and go through a tightly regulated developmental program of proliferation, hypertrophy, and apoptosis to be eventually replaced by osteoblasts in the ossification centres.[59] IHH signalling may also regulate osteoblast differentiation during endochondral bone development in interaction with WNT/β-catenin.[60] Within this cross-pathway signalling there are also several GWAS-identified genes such as *WNT1, WNT4, WNT5b, WNT16, DHH*, and *PTCH1*. The latter gene encodes the patched−1 receptor (Ptch1) which is essential for many developmental processes such as osteoblastogenesis and chondrocyte differentiation.[61] Pat-ched1 haploinsufficiency (Ptch1+/−) is characterized by increased adult bone mass in mice; while in culture cells exhibited accelerated osteoblast differentiation.[61]

OPG–RANK–RANKL signalling

The OPG–RANK–RANKL signalling pathway predominantly regulates the coupling between osteoblasts and osteoclast activity [63]. Osteoblasts secrete the receptor activator of NF-κB ligand (RANKL) which binds to its receptor activator of NF-κB (RANK) on monocytes resulting in osteoclast differentiation and activation the presence of monocyte colony-stimulating factor (M-CSF). OPG also secreted by the osteoblast is a decoy receptor of RANKL and blocks osteoclast induction by competing with RANK to bind RANKL. These factors are part of the tumour necrosis factor (TNF) superfamily of ligands and receptors and have been shown to have other functions beyond bone remodelling, including potential roles in other disease processes (i.e., vascular calcification, diabetes and cancer). *TNFRSF11 (RANKL), TNFRSF11A (RANK)* and *TNFSF11B (OPG)* were also one of the first genes associated with osteoporosis found and replicated by several studies. Functional studies have shown that in RANK−/− mice the generation of osteoclasts from their myeloid progenitors is blocked resulting in absence of bone

resorption and severe osteopetrosis. [64] Although this pathway has not been associated with fracture risk it holds an important role in increasing BMD and decreasing fracture risk as due to its antiresorptive effects discuses below.

THE FULFILLED AND UNFULFILLED PROMISES OF GENOMICS

The ultimate goal of osteoporosis genomics after GWAS is to perform functional validation, that will allow translating the discoveries into clinical practice. It has been shown that genetic information may significantly improve the search of drug targets [65] and may increase the success rate of preclinical and clinical trials. Nowadays, most of the osteoporosis agents in use (or undergoing trials) target pathways related to the discovered BMD genes. Denosumab is a human monoclonal antibody which binds to RANKL and inhibits bone resorption by preventing RANKL from activating RANK, its receptor on the osteoclast surface. Such mimicking of the decoy action of OPG reduces the formation, activation, and survival of osteoclasts;[66] This RANKL inhibitor is approved for use in postmenopausal women with risk of osteoporosis. Since its approval, it has shown sustained efficacy in increasing BMD and decreasing vertebral fracture risk. [67] Anabolic agents hold promising potential, constituting bone-building drugs with proven success, such as PTH 1-34 (teriparatide) and PTHrP 1-36 (abaloparatide), which stimulate osteoblasts to make new bone. GWAS have identified genes in the PTH pathway including *PTHLH*.[20] Teriparatide, is the first anabolic agent approved for the treatment of osteoporosis. In clinical trials to date, abaloparatide has shown promising results in a reduction of new-onset vertebral (approximately 86% reduction) and nonvertebral fractures (approximately 43% reduction).[68] Finally, several GWAS on BMD and fracture risk have identified variants in several genes (SOST-sclerostin, *DKK1, LRP5, LRP4, AXIN1,* and *CTNNB1*-β-catenin) in pathways with anabolic potential.[20] Sclerostin (which is produced by osteocytes) inhibits the proliferation, differentiation, and survival of osteoblasts, leading to reduced bone formation. Sclerostin also stimulates (in neighboring osteocytes) the production of RANKL, leading to bone resorption. Anti-sclerostin antibodies, Romosozumab and Blosozumab, have been developed to counterattack the sclerostin effects and have undergone phase II clinical trials.[69] Only Romosozumab was followed to phase III where it showed a 73% reduction in vertebral fracture risk and a 36% reduction in clinical fractures at 1 year (reviewed by Reid et al).[70] Overall the current agents have been mainly successful in reducing the risk of vertebral fractures (up to 70%), whereas reduction rates for non-vertebral fractures and hip fractures were of smaller magnitude (20% and 40%, respectively);[71] indicating that trabecular and cortical bone might be regulated by different biological pathways or respond differently to

signals involved in the regulation of skeletal homeostasis, as recently shown by the WNT16 contrasting mechanisms of action.[52,57]

Identification of causal genes for bone traits

It is a common approach to annotate the closest gene to a SNP with the lowest p-value as the most likely causal gene. However, it is important to note that the physical distance of a variant to a gene is not substantive evidence of causality.[73] Multitude of statistical and functional fine-mapping methods have been developed in order to prioritize causal variants (reviewed by Spain et al) .[73] These methods are essential for highly polygenic traits such as bone density and can drastically improve the mapping of associated loci to their causal genes. For example, in the latest and largest GWAS to date in the field of osteoporosis, Morris et al. [25] have used UK Biobank data to build a robust analysis pipeline to prioritize eBMD-associated SNPs by combining several distinctive approaches. Firstly, two statistical fine-mapping methods were used to refine associated SNPs at each locus. These methods identify SNPs based on their conditional independence (GCTA-COJO) and posterior probability (log10 Bayes factor > 3) for causality (FINEMAP); yielding two conditionally independent SNPs and five SNPs with a log10 Bayes factor > 3 per locus. Second, all fine-mapped SNPs were then tested for enrichment for missense protein coding SNPs, DHSs (DNase I hypersensitive sites) from primary osteoblasts, and ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) peaks from SaOS-2 osteosarcoma cells. Notably, the fine-mapped SNPs showed strong enrichment for both missense variants in protein-coding regions and osteoblast open chromatin sites. Finally, a list of positive control genes identified through pharmacotherapy or Mendelian disease which are critical for bone biology were curated. Six distinctive genomic features that link a SNP to a gene such as cell-specific 3-dimensional (3D) contact domains, cell-specific open chromatin states, physical proximity, and the presence of associated coding variation were used to construct Target Gene sets which were tested for enrichment for positive control bone genes. All six methods for linking fine-mapped SNPs to Target Genes yielded strong enrichment for positive control genes known to be central to bone biology.[25] Notably, Target Genes closest to fine-mapped SNPs in osteoblast-derived ATAC-seq peaks were seen to be ~60-fold enriched for positive control genes. This is quite an insightful finding, as transcription factor binding occurs mainly in regions of open chromatin (ATAC-seq peaks), thus, this implies that the mapped Target Genes may have substantial impact on bone biology.

POST-GWAS ANALYSES AND CONCLUDING REMARKS

In the fast-moving world of genomics, including the field of osteoporosis, hundreds of genetic markers have been identified as associated with complex traits. As discussed above the GWAS findings have aided the discovery of several novel osteoporosis drug targets. However, there is still an overwhelming amount of significant associations, for which the underlying biological mechanisms remain unknown; as the functional characterization of the discovered genetic variants have lagged far behind.[74] In order to understand the functional consequences of these loci future post-GWAS methods should be focused on bridging the gap between disease-associated loci and underlying disease biology.[74] An essential step in the translation studies, from gene discovery to biological mechanisms, is the identification of causal variants and genes. It is well known that the most strongly associated variants with a specific trait or diseases are likely to be in linkage disequilibrium (LD) with the causal variant, rather than have a biological function themselves.[73] In the last few years large numbers of studies have been devoted to pinpoint causal variants using both statistical evidence (e.g. reference panels, targeted resequencing, Bayesian methods, machine learning methods) from large association data sets and functional annotations of genetic variants (e.g. enrichment analysis, pathway prioritization, DNase sensitivity).[73,75] Moreover, the emergence of expression quantitative trait loci (eQTLs), which characterize associations between genetic variants and gene expression at the cellular level, have provided a better biological context in disease studies.[76] Although a widely used approach, eQTL data from primary bone cells is limited. Establishing bone specific eQTLs may significantly improve the search of causal variants and provide valuable target genes. Finally, the identification of causal variants can further facilitate the interpretation of the GWAS findings and opens opportunities for more detailed downstream functional investigations such as in human and/or animal cell and tissues models.

GWAS have provided us with a comprehensive understanding of the genetic architecture of osteoporosis and fracture risk. Moreover, key bone genes and pathways have been identified which have promoted novel drug targets and treatments. In the decade to come, the advances of GWAS and post-GWAS techniques and methods will enable fruitful incorporation of genetics in clinical practice that will ensure better disease prediction and risk stratification, leading to overall improvement in disease prevention or intervention.

REFERENCES

1. G.S. Keene, M.J. Parker, G.A. Pryor, Mortality and morbidity after hip fractures, BMJ 307 (6914) (1993) 1248–1250.
2. J. Panula, H. Pihlajamäki, V.M. Mattila, P. Jaatinen, T. Vahlberg, P. Aarnio, et al., Mortality and cause of death in hip fracture patients aged 65 or older: a population-based study, BMC Musculoskelet. Disord. 12 (2011) 105
3. J.D. Adachi, S. Adami, S. Gehlbach, F.A. Anderson, S. Boonen, R.D. Chapurlat, et al., Impact of prevalent fractures on quality of life: baseline results from the global longitudinal study of osteoporosis in women, Mayo Clin. Proc. 85 (9) (2010 Sep) 806–813.
4. F. Rivadeneira, O. Mäkitie, Osteoporosis and bone mass disorders: from gene pathways to treatments, Trends Endocrinol. Metab. 27 (5) (2016) 262–281.
5. N.K. Arden, J. Baker, C. Hogg, K. Baan, T.D. Spector, The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of post-menopausal twins, J. Bone Miner. Res. 11 (4) (2009 Dec 3) 530–534.
6. S. Demissie, J. Dupuis, L.A. Cupples, T.J. Beck, D.P. Kiel, D. Karasik, Proximal hip geometry is linked to several chromosomal regions: genome-wide linkage results from the Framingham osteoporosis study, Bone 40 (3) (2007) 743–750
7. D. Karasik, S. Demissie, Y. Zhou, D. Lu, K.E. Broe, M.L. Bouxsein, et al., Heritability and genetic correlations for bone microarchitecture: the Framingham study fa-milies, J. Bone Miner. Res. 32 (1) (2017) 106–114
8. K. Trajanoska, J.D. Schoufour, E.A.L. de Jonge, B.C.T. Kieboom, M. Mulder, B.H. Stricker, et al., Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: the Rotterdam study, Bone 114 (2018) 116–124
9. C.J. Hernandez, M.C. van der Meulen, Understanding bone strength is not enough, J. Bone Miner. Res. 32 (6) (2017 Jun) 1157–1162
10. D.P. Kiel, S. Demissie, J. Dupuis, K.L. Lunetta, J.M. Murabito, D. Karasik, Genome-wide association with bone mass and geometry in the Framingham heart study, BMC Med Genet 8 (Suppl. 1) (2007) S14
11. P.M. Visscher, N.R. Wray, Q. Zhang, P. Sklar, M.I. McCarthy, M.A. Brown, et al., 10 years of GWAS discovery: biology, function, and translation, Am. J. Hum. Genet. 101 (1) (2017 Jul 6) 5–22
12. J. Richards, F. Rivadeneira, M. Inouye, T. Pastinen, N. Soranzo, S. Wilson, et al., Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide as-sociation study, Lancet 371 (9623) (2008) 1505–1512 May.
13. J.B.J. van Meurs, T.A. Trikalinos, S.H. Ralston, S. Balcells, M.L. Brandi, K. Brixen, et al., Large-scale analysis of association between <emph type="ital& quot;>LRP5</emph> and <emph type="ital">LRP6</emph> variants and osteoporosis, JAMA 299 (11) (2008) 1277
14. U. Styrkarsdottir, B.V. Halldorsson, S. Gretarsdottir, D.F. Gudbjartsson, G.B. Walters, T. Ingvarsson, et al., Multiple genetic loci for bone mineral density and fractures, N. Engl. J. Med. 358 (22) (2008) 2355–2365 May.
15. U. Styrkarsdottir, B.V. Halldorsson, S. Gretarsdottir, D.F. Gudbjartsson, G.B. Walters, T. Ingvarsson, et al., New sequence variants associated with bone mineral density, Nat. Genet. 41 (1) (2008) 15–17 Dec.

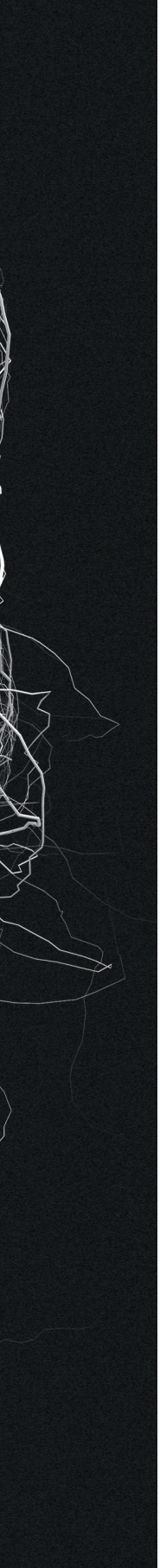
16. N.J. Timpson, J.H. Tobias, J.B. Richards, N. Soranzo, E.L. Duncan, A.-M. Sims, et al., Common variants in the region around Osterix are associated with bone mineral density and growth in childhood, Hum. Mol. Genet. 18 (8) (2009) 1510–1517
17. C. Medina-Gomez, J.P. Kemp, K. Estrada, J. Eriksson, J. Liu, S. Reppe, et al., Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus, PLoS Genet. 8 (7) (2012) e1002718 Jul.
18. H.-F.F. Zheng, J.H. Tobias, E. Duncan, D.M. Evans, J. Eriksson, L. Paternoster, et al., WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk, PLoS Genet. 8 (7) (2012 Jul) e1002745.
19. F. Rivadeneira, U. Styrkársdottir, K. Estrada, B.V. Halldórsson, Y.-H. Hsu, J.B. Richards, et al., Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies, Nat. Genet. 41 (11) (2009)1199–1206 Oct.
20. K. Estrada, U. Styrkarsdottir, E. Evangelou, Y.-H. Hsu, E.L. Duncan, E.E. Ntzani, et al., Genome-wide meta-analysis identifies 56 bone mineral density loci and re-veals 14 loci associated with risk of fracture, Nat. Genet. 44 (5) (2012) 491–501. Apr.
21. C. Ohlsson, H. Wallaschofski, K.L. Lunetta, L. Stolk, J.R.B. Perry, A. Koster, et al., Genetic determinants of serum testosterone concentrations in men. Abecasis GR, editor, PLoS Genet. 7 (10) (2011) e1002313
22. H. Zheng, V. Forgetta, Y.-H. Hsu, K. Estrada, A. Rosello-Diez, P.J. Leo, et al., Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture, Nature 526 (7571) (2015) 112–117 Sep.
23. C. Medina-Gomez, J.P. Kemp, K. Trajanoska, J. Luan, A. Chesi, T.S. Ahluwalia, et al., Life-course genome-wide association study meta-analysis of Total body BMD and assessment of age-specific effects, Am. J. Hum. Genet. 102 (1) (2018 Jan 4) 88–102
24. J.P. Kemp, J.A. Morris, C. Medina-Gomez, V. Forgetta, N.M. Warrington, S.E. Youlten, et al., Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis, Nat. Genet. 49 (10) (2017) 1468–1475
25. J.A. Morris, J.P. Kemp, S.E. Youlten, L. Laurent, J.G. Logan, R.C. Chai, et al., An atlas of genetic influences on osteoporosis in humans and mice, Nat. Genet. 51 (2) (2019) 258–266
26. U. Styrkarsdottir, G. Thorleifsson, P. Sulem, D.F. Gudbjartsson, A. Sigurdsson, A. Jonasdottir, et al., Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits, Nature 497 (7450) (2013) 517–520
27. C.L. Gregson, F. Newell, P.J. Leo, G.R. Clark, L. Paternoster, M. Marshall, et al., Genome-wide association study of extreme high bone mass: contribution of common genetic variation to extreme BMD phenotypes and potential novel BMD-associated genes, Bone 114 (2018) 62–71
28. L. Oei, K. Estrada, E.L. Duncan, C. Christiansen, C.-T. Liu, B.L. Langdahl, et al., Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus, Bone 59 (2014 Feb) 20–27
29. N. Alonso, K. Estrada, O.M.E. Albagha, L. Herrera, S. Reppe, O.K. Olstad, et al., Identification of a novel locus on chromosome 2q13, which predisposes to clinical vertebral fractures independently of bone density, Ann. Rheum. Dis. 77 (3) (2018) 378–385
30. Y. Guo, L.-J. Tan, S.-F. Lei, T.-L. Yang, X.-D. Chen, F. Zhang, et al., Genome-wide association study identifies ALDH7A1 as a novel susceptibility gene for osteo-porosis, Georges M, editor, PLoS Genet. 6 (1) (2010 Jan 8) e1000806

31. K. Trajanoska, J.A. Morris, L. Oei, H.-F. Zheng, D.M. Evans, D.P. Kiel, et al., Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study, BMJ 362 (2018 Aug 29) k3225
32. L. Paternoster, M. Lorentzon, L. Vandenput, M.K. Karlsson, Ö. Ljunggren, A. Kindmark, et al., Genome-wide association meta-analysis of cortical bone mi-neral density unravels allelic heterogeneity at the RANKL locus and potential pleiotropic effects on bone. Gibson G, editor, PLoS Genet. 6 (11) (2010) e1001217
33. L. Paternoster, M. Lorentzon, T. Lehtimäki, J. Eriksson, M. Kähönen, O. Raitakari, et al., Genetic determinants of trabecular and cortical volumetric bone mineral densities and bone microstructure, Richards JB, editor, PLoS Genet. 9 (2) (2013 Feb 21) e1003247
34. C.M. Nielson, C.-T. Liu, A.V. Smith, C.L. Ackert-Bicknell, S. Reppe, J. Jakobsdottir, et al., Novel genetic variants associated with increased vertebral volumetric BMD, reduced vertebral fracture risk, and increased expression of SLC1A3 and EPHB2, J. Bone Miner. Res. 31 (12) (2016) 2085–2097
35. Y.-H. Hsu, M.C. Zillikens, S.G. Wilson, C.R. Farber, S. Demissie, N. Soranzo, et al., An integration of genome-wide association study and gene expression profiling toprioritize the discovery of novel susceptibility loci for osteoporosis-related traits, PLoS Genet. 6 (6) (2010 Jun 10) e1000977
36. D.A. Baird, D.S. Evans, F.K. Kamanu, J.S. Gregory, F.R. Saunders, C.V. Giuraniuc, et al., Identification of novel loci associated with hip shape: a meta-analysis of genome-wide association studies, J. Bone Miner. Res. 34 (2) (2019 Feb) 241–251
37. N.R. Wray, M.E. Goddard, P.M. Visscher, Prediction of individual genetic risk to disease from genome-wide association studies, Genome Res. 17 (10) (2007) 1520–1528
38. C. Valero, M.T. Zarrabeitia, J.L. Hernandez, A. Zarrabeitia, J. Gonzalez-Macias, J.A. Riancho, Bone mass in young adults: relationship with gender, weight and genetic factors, J. Intern. Med. 258 (6) (2005) 554–562
39. A. Cheng, P.G. Genever, SOX9 determines RUNX2 transactivity by directing in-tracellular degradation, J. Bone Miner. Res. 25 (12) (2010) 2680–2689
40. J.B. Lian, G.S. Stein, A. Javed, A.J. van Wijnen, J.L. Stein, M. Montecino, et al., Networks and hubs for the transcriptional control of osteoblastogenesis, Rev. Endocr. Metab. Disord. 7 (1–2) (2006) 1–16
41. J.P. Stains, R. Civitelli, Genomic approaches to identifying transcriptional reg-ulators of osteoblast differentiation, Genome Biol. 4 (7) (2003) 222
42. X. Yang, G. Karsenty, Transcription factors in bone: developmental and pathological aspects, Trends Mol. Med. 8 (7) (2002 Jul 1) 340–345
43. K. Nakashima, B. de Crombrugghe, Transcriptional mechanisms in osteoblast dif-ferentiation and bone formation, Trends Genet. 19 (8) (2003) 458–466
44. V. Krishnan, H.U. Bryant, O.A. Macdougald, Regulation of bone mass by Wnt sig-naling, J. Clin. Invest. 116 (5) (2006 May) 1202–1209
45. Y. Gong, R.B. Slee, N. Fukai, G. Rawadi, S. Roman-Roman, A.M. Reginato, et al., LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development, Cell 107 (4) (2001) 513–523
46. R.D. Little, C. Folz, S.P. Manning, P.M. Swain, S.-C. Zhao, B. Eustace, et al., A mutation in the LDL receptor–related protein 5 gene results in the autosomal dominant high–bone-mass trait, Am. J. Hum. Genet. 70 (1) (2002 Jan) 11–19

47. L. Van Wesenbeeck, E. Cleiren, J. Gram, R.K. Beals, O. Bénichou, D. Scopelliti, et al., Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density, Am. J. Hum. Genet. 72 (3) (2003 Mar) 763–771
48. M. Ai, S. Heeger, C.F. Bartels, D.K. Schelling, Osteoporosis-Pseudoglioma Collaborative Group. Clinical and molecular findings in osteoporosis-Pseudoglioma syndrome, Am. J. Hum. Genet. 77 (5) (2005 Nov) 741–753
49. L.M. Boyden, J. Mao, J. Belsky, L. Mitzner, A. Farhi, M.A. Mitnick, et al., High bone density due to a mutation in LDL-receptor–related protein 5, N. Engl. J. Med. 346 (20) (2002 May 16) 1513–1521
50. D.L. Koller, H.-F. Zheng, D. Karasik, L. Yerges-Armstrong, C.-T. Liu, F. McGuigan, et al., Meta-analysis of genome-wide studies identifies *WNT16* and *ESR1* SNPs as-sociated with bone mineral density in premenopausal women, J. Bone Miner. Res. 28 (3) (2013 Mar) 547–558
51. S. Movérare-Skrtic, P. Henning, X. Liu, K. Nagano, H. Saito, A.E. Börjesson, et al., Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures, Nat. Med. 20 (11) (2014 Nov 12) 1279–1288
52. S. Movérare-Skrtic, J. Wu, P. Henning, K.L. Gustafsson, K. Sjögren, S.H. Windahl, et al., The bone-sparing effects of estrogen and WNT16 are independent of each other, Proc. Natl. Acad. Sci. U. S. A. 112 (48) (2015) 14972–14977
53. M.A. Arnold, Y. Kim, M.P. Czubryt, D. Phan, J. McAnally, X. Qi, et al., MEF2C transcription factor controls chondrocyte hypertrophy and bone development, Dev. Cell 12 (3) (2007) 377–389 C.M. Laine, K.S. Joeng, P.M. Campeau, R. Kiviranta, K. Tarkkonen, M. Grover, et al., WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta, N. Engl. J. Med. 368 (19) (2013) 1809–1816
54. K.S. Joeng, Y.-C. Lee, J. Lim, Y. Chen, M.-M. Jiang, E. Munivez, et al., Osteocyte-specific WNT1 regulates osteoblast function during bone homeostasis, J. Clin. Invest. 127 (7) (2017) 2678–2688
55. R.A. Deckelbaum, A. Majithia, T. Booker, J.E. Henderson, C.A. Loomis, The ho-meoprotein engrailed 1 has pleiotropic functions in calvarial intramembranous bone formation and remodeling, Development 133 (1) (2006 Jan 1) 63–74
56. J. Regan, F. Long, Notch signaling and bone remodeling, Curr Osteoporos Rep 11(2) (2013) 126–129
57. R. Kopan, M.X.G. Ilagan, The canonical Notch signaling pathway: unfolding the activation mechanism, Cell 137 (2) (2009 Apr 17) 216–233
58. J. Yang, P. Andre, L. Ye, Y.-Z. Yang, The Hedgehog signalling pathway in bone formation, Int J Oral Sci 7 (2) (2015 Jun 29) 73–79
59. K.K. Mak, M.-H. Chen, T.F. Day, P.-T. Chuang, Y. Yang, Wnt/-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation, Development 133 (18) (2006) 3695–3707
60. F. Long, Building strong bones: molecular regulation of the osteoblast lineage, Nat Rev Mol Cell Biol 13 (1) (2012) 27–38
61. S. Ohba, H. Kawaguchi, F. Kugimiya, T. Ogasawara, N. Kawamura, T. Saito, et al., Patched1 Haploinsufficiency increases adult bone mass and modulates Gli3 re-pressor activity, Dev. Cell 14 (5) (2008 May 13) 689–699
62. S. Zhang, X. Wang, G. Li, Y. Chong, J. Zhang, X. Guo, et al., Osteoclast regulation of osteoblasts via RANK-RANKL reverse signal transduction in vitro, Mol. Med. Rep. 16 (4) (2017) 3994–4000

63. W.C. Dougall, M. Glaccum, K. Charrier, K. Rohrbach, K. Brasel, T. De Smedt, et al., RANK is essential for osteoclast and lymph node development, Genes Dev. 13 (18) (1999) 2412–2424
64. M.R. Nelson, H. Tipney, J.L. Painter, J. Shen, P. Nicoletti, Y. Shen, et al., The support of human genetic evidence for approved drug indications, Nat. Genet. 47a(2015) 856–860
65. P.J. Kostenuik, H.Q. Nguyen, J. McCabe, K.S. Warmington, C. Kurahara, N. Sun, et al., Denosumab, a fully human monoclonal antibody to RANKL, inhibits bone resorption and increases BMD in Knock-in mice that express chimeric (murine/ human) RANKL*, J. Bone Miner. Res. 24 (2) (2009) 182–195
66. S. Zaheer, M. LeBoff, E.M. Lewiecki, Denosumab for the treatment of osteoporosis, Expert Opin. Drug Metab. Toxicol. 11 (3) (2015) 461–470.
67. S.H. Tella, A. Kommalapati, R. Correa, Profile of Abaloparatide and its potential in the treatment of postmenopausal osteoporosis, Cureus 9 (5) (2017 May 31) e1300
68. R.R. Recker, C.T. Benson, T. Matsumoto, M.A. Bolognese, D.A. Robins, J. Alam, et al., A randomized, double-blind phase 2 clinical trial of Blosozumab, a Sclerostin antibody, in postmenopausal women with low bone mineral density, J. Bone Miner. Res. 30 (2) (2015) 216–224
69. I.R. Reid, Targeting Sclerostin in postmenopausal osteoporosis: focus on Romosozumab and Blosozumab, BioDrugs 31 (4) (2017) 289–297 Internet. Aug 25 [cited 2018 Dec 3].
70. J.S. Chen, P.N. Sambrook, Antiresorptive therapies for osteoporosis: a clinical overview, Nat Rev Endocrinol 8 (2) (2012 Feb 6) 81–91
71. C. Ohlsson, P. Henning, K.H. Nilsson, J. Wu, K.L. Gustafsson, K. Sjögren, et al., Inducible *Wnt16* inactivation: WNT16 regulates cortical bone thickness in adult mice, J. Endocrinol. 237 (2) (2018 May) 113–122
72. S.L. Spain, J.C. Barrett, Strategies for fine-mapping complex traits, Hum. Mol. Genet. 24 (R1) (2015) R111–R119 Internet. Oct 15 [cited 2018 Dec 5].
73. M.D. Gallagher, A.S. Chen-Plotkin, The post-GWAS era: from association to func-tion, Am. J. Hum. Genet. 102 (5) (2018 May 3) 717–730
74. D.J. Schaid, W. Chen, N.B. Larson, From genome-wide associations to candidate causal variants by statistical fine-mapping, Nat Rev Genet 19 (8) (2018 Aug 29) 491–504
75. A.C. Nica, E.T. Dermitzakis, Expression quantitative trait loci: present and future, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 368 (1620) (2013) 20120362
76. S.K. Kim, Identification of 613 new loci associated with heel bone mineral density and a polygenic risk score for bone mineral density, osteoporosis and fracture, PLoS One 13 (7) (2018) e0200785.





2.2.2

Genome-wide association meta-analysis identifies seven loci for osteocalcin levels

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ABSTRACT

Osteocalcin, the most abundant noncollagenous bone matrix protein involved in bone mineralization and bone turnover, is encoded by the BGLAP gene; however, there is no data on the influence of common genetic variation on osteocalcin levels. We performed a genome-wide association study for osteocalcin levels including 20,922 European individuals. Genome-wide associations were tested using natural log transformed osteocalcin levels adjusting for age, age^2, sex, BMI and ancestral genetic background estimated by PCA. Inverse variance meta-analysis was performed, and significance was set at P≤5x10^{-8}. LD-score regression was used to estimate heritability and genetic correlation (shared heritability) with other traits. SNP-based heritability was estimated as 0.09 (SE: 0.02). Variants at seven loci reached genome-wide significance (GWS): 1q22 (*SMG5,* p=3.66x10^{-25}), 8q24.12 (*TNFRSF11B*, p=3.72x10^{-22}), 11q13.1 *(SSSCA1-AS1*, 1.75x10^{-9}), 11q24.3 (*FLI1*, p=5.68x10^{-9}), 13q14.11 (AKAP11, p=8.2x10^{-18}) 16p13.3 (TSC2, p=7.24x10^{-9}) and 19q13.41 (*FPR3,* p=1.01x10-8); after validation in five cohorts (n=9,467). Osteocalcin had modest negative genetic correlations (shared heritability) with BMD (femoral neck BMD rg:-0.27, se=0.10; lumbar spine BMD rg:-0.35, se:0.12), type 2 diabetes (rg:-0.26, se:0.11) and several metabolic traits and diseases such as waist-to-hip ratio (rg:-0.27, se:0.09) and triglycerides (rg:-0.27, se:0.08). MR analysis did not show any causal effect of osteocalcin on several glycemic traits. The results of this study highlight the polygenicity of serum osteocalcin levels and offers insights into its regulation in humans. Although osteocalcin was genetically correlated with several metabolic traits its causal role was not confirmed.

INTRODUCTION

Osteocalcin (gamma-carboxyglutamic acid protein, BGLAP), a bone-derived hormone, comprises about 15% of the noncollagenous protein component of the extracellular bone matrix and is the most abundant noncollagenous protein of the human body.[1] Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and reflects the rate of bone formation.[2] Currently, it is used as a non-invasive bone formation biomarker for the clinical assessment of calcium-phosphorus metabolic disturbances of bone in diseases or drug trials.[2]

The osteocalcin protein contains gamma carboxyglutamate (Gla) residues which are carboxylated by the enzyme gamma glutamyl carboxylase, a process that is highly dependent to vitamin K. The carboxylated osteocalcin is secreted from the osteoblasts and incorporated in the bone matrix by binding to hydroxyapatite in a calcium-dependent fashion. Some of its calcium-binding properties led to the suggestion that osteocalcin is involved in the bone matrix mineralization.[3] The extracellular bone matrix provides elasticity and flexibility to bone and also determines its structural organization. Uncarboxylated or undercarboxylated osteocalcin residues cannot be incorporated in the matrix and are release into the circulation. Intact serum osteocalcin reflects the portion of newly synthesized osteocalcin that does not bind to the mineral phase of the bone but is released directly into the circulation.

In addition to its involvement in bone formation, osteocalcin has been suggested to play an important role in endocrine regulation of energy metabolism,[5] male fertility,[6] exercise capacity,[7] brain development,[8] and age-related decline in cognition.[9] Studies suggested that osteocalcin regulates glucose metabolism, insulin secretion and adiponectin expression via directly interacting with β-cells in the pancreas and adipocytes in the adipose tissues as a feedback loop of Leptin's regulation of osteoblasts.[10,11] Osteocalcin-deficient mice are obese with decreased β-cell proliferation and greater insulin resistance.[5] Osteocalcin-treated deficient mice had a significantly decrease in blood glucose and an increased insulin secretion. Moreover, wild type mice treated with non-carboxylated osteocalcin improved insulin sensitivity (partially through the enhancement of β-cell proliferation); increased β-cell insulin secretion, energy expenditure and adiponectin expression; and were protected against the development of obesity and type 2 diabetes (T2DM) induced by high-fat diet or hyperphagia.[12]

Although, as described above, strong evidences demonstrated osteocalcin plays an important role in endocrine regulation of energy metabolism; epidemiological studies, on the other hand, have not been conclusive. Low concentrations of baseline serum osteocalcin were strongly associated with an increased risk of incident diabetes during a 5-year follow-up.[13] In incident diabetic subjects, concentrations of serum osteocalcin were inversely associated with insulin resistance (HOMA-IR). In non-diabetic

men exposure to higher plasma osteocalcin during a 3-year follow-up was associated with a significantly lower rise in fasting plasma glucose.[14] However, no correlation between baseline serum osteocalcin and the incidence of diabetes was observed after a 3-year follow-up in a high-risk cohort with T2D.[15] In addition, bisphosphonate treatment for osteoporosis reduced both carboxylated and non-carboxylated forms of osteocalcin, but no association was found between these changes and glucose parameters after 16 weeks of treatment in subjects with impaired fasting glucose.[16] The variation of serum osteocalcin due to environmental and/or genetic factors may confound the findings from observation studies. For example, an increase of 16%-70% in serum osteocalcin in winter compared with that of summer was found in some studies.[17,18] Serum osteocalcin appears to be slightly higher in males at all ages. [19,20] Whereas an age-related decline of serum osteocalcin 19 positively correlated with estradiol concentration, was observed in women. [21]

The estimated heritability of serum osteocalcin was 62% in a Mexican American population, suggesting that the variation of serum osteocalcin may also be due to genetic factors. [22] A common SNP, rs1800247, located at the 5' UTR of the osteocalcin encoded gene, bone gamma-carboxyglutamate protein, BGLAP, was reported to be associated with lower serum osteocalcin,[23] but didn't replicate in other studies.[24,25] Given that the BGLAP gene doesn't seem to explain the variability of serum osteocalcin, seeking to identify polygenetic factors that contribute to serum osteocalcin variation and to causally infer relations between serum osteocalcin and its effect on metabolic disorders, we conducted a GWAS meta-analysis on 13 studies of European-descent adults and replicated top genome-wide associated findings in additional five independent studies. The replicated SNPs were used as instrumental variables for Mendelian randomization (MR) analyses to examine the causal effect of osteocalcin on metabolic and diabetic relevant disorders.

SUBJECTS AND METHODS

Study Subjects, Genotyping and Imputation

The Discovery stage of this GWAS meta-analysis utilized data from 11 cohort studies, including AGEs, Framingham, GOOD, HealthABC (European Ancestry), Mr.OS Sweden, Mr.OS US, PIVUS, SHIP, SHIP-Trend, Rotterdam study (I, II and II) and TwinsUK cohorts. A total of 20,922 European-descent adults were included in the final analyses. The independent replication of top associated findings, including genome-wide associated SNPs (association p-values $\leq 5x10^{-8}$) and genome-wide suggestive associated SNPs ($5x10^{-8} \leq$ association p-values $\leq 5x10^{-6}$), were performed in five additional studies, including DECODE, BPROOF, GINLISA, Health2006 and OPRA cohorts. A total of 9,467

adult European-descent adults were included in the replication analyses. Distribution of age, sex, BMI and T2DM prevalence in the participating discovery and replication cohorts is available in the Supplementary Materials (**Supplementary table 1**). All studies were approved by institutional ethics review committees at the relevant organizations and all participants provided written informed consent.

All the cohorts were genotyped using commercially available SNP arrays from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) or Illumina (Illumina Inc., San Diego, CA, USA). The detail of genotype arrays, calling algorithms, quality controls for genotyping, inclusion criteria of SNPs and subjects is available in the Supplementary Materials (Table S2). Imputation was performed within each study with IMPUTE,[26] IMPUTE2,[27] MACH[28] or Minimac[29] software (**Supplementary table 2**) using genotypes from the HapMap Phase II release 22, NCBI build 37 (CEU samples) or HRC1.1[30] as reference panels. Before performing an analysis, poorly imputed and low frequency or rare polymorphisms were excluded. Specifically, the quality control filters applied for exclusions of SNPs were: imputation quality score <0.3 for MACH/Minimac and <0.4 for IMPUTE/IMPUTE2, average minor allele frequency (MAF) of <1% across studies, and SNPs missing from ≥50% of the cohorts. After quality control, ~2.5 million SNPs were available from each cohort for the discovery meta-analysis.

Serum Osteocalcin Measurement

Serum total osteocalcin was measured by assays with different specificities including chemiluminescence assay, eletrochemilumin-escence immunoassay, ELISA, EIA and RIA. The intact osteocalcin is 49 amino acid long. The N-terminal part of the osteocalcin includes molecule fragment from amino acid#1 to amino acid#19. The C-terminal part of the osteocalcin includes molecule fragment from amino acid#44 to amino acid#49. The middle "mid" part of the osteocalcin includes molecule fragment from amino acid#20 to amino acid#43. The majority of these assays capture the intact osteocalcin and/or large N-terminal-mid molecule (fragment amino acid #1 to amino acid#43). For example, RIA was used to measure serum osteocalcin in Framingham Study participants. This RIA assay captures both intact osteocalcin (49 amino acids) and the large N-terminal-mid molecule fragment (amino acid#1 to amino acid#43). Given these differences the serum osteocalcin levels may not be directly comparable across studies. As showed in **Supplementary table 1**, the average serum osteocalcin level in men is 26.3, 16.5 and 4.4, respectively, in participants from GOOD Study (using ELISA), MROS (using EIA) and Framingham Study (using RIA). The distribution of serum osteocalcin across studies as well as the assays they used are available in the Supplementary Materials (**Supplementary table 1**). The non-carboxylated form of osteocalcin can be further separated from the carboxylated form. As showed in Table S1, the % of the non-carboxylated osteocalcin among total serum osteocalcin is 18%,

34% and 19%, respectively, in Framingham, HealthABC and MROS US studies. Since the other studies didn't measure non-carboxylated osteocalcin, we only perform GWAS analyses on the total osteocalcin level.

SNP-Osteocalcin association Analyses

In the Discovery and replication phases, each cohort conducted analyses according to a standard pre-specified analysis plan under an additive (i.e. per allele count) genetic model. Both sex-specific and sex-combined analyses were performed. The phenotype was log2 transformed serum total osteocalcin level. The covariate adjustment in the model included sex (only for sex-combined models), age, age2, BMI and ancestral genetic background estimated by PCA. For family-based studies with pedigree structures, such as the Framingham Study, a mixed-effect model to take into account the within familiar correlation was applied. The mixed-effect model was implemented in the R Kinship2 package (https://cran.rproject.org/web/packages/available_packages_by_name.html). Study-specific summary statistics (beta-coefficients, standard errors, p-values and sample size) were used for the meta-analysis with the genome-wide SNPs.

Meta-Analysis

Meta-analysis of the GWAS discovery results was conducted and tested independently by two researchers. Due to the relatively limited statistical power to detect sex-specific associations, we only present results from sex-combined meta-analysis. Given the relative incomparable serum total osteocalcin measurements across studies, we applied a fixed-effect, sample size weighted meta-analysis implemented in METAL software. λ genomic control was estimated and applied to meta-analysis to correct for potential inflation of the test statistics. The QQ plot was generated by R package (EasyQQ). The regional plots were generated by LocusZoom, [31] using chromosome position coordinates as provided in GrCh37/hg19. I^2 was estimated per SNP to quantify heterogeneity across studies. We consider genome-wide level of statistical significance at p-value $\leq 5\times10^{-8}$ and the suggestive level of significance at p-value $\leq 5\times10^{-6}$.

Conditional Analyses for Independent Signals

To identify independent association signals in the regions containing SNPs that were genome-wide significant or genome-wide suggestively significant, we performed chromosomal-wide association analyses conditioning on the most significantly associated SNP(s) from locus (loci) on the same chromosome. This was done by using variance-component models implemented in the GCTA (Genome-wide Complex Trait Analysis) tool package[32] with summary statistics from discovery meta-analysis along

with LD information from HRC1.1 using the BPROOF study.[33] Conditionally independent variants were then annotated to the closest gene using the Ensembl annotation system implemented by SNP-NEXUS (https://snp-nexus.org/index.html).

Replication Analysis

In each genome-wide associated locus, suggestive associated locus or genome-wide associated independent signals in the HapMap effort we selected the lead SNP with the lowest association p-value for de-novo replication in three cohorts. In addition, SNP with $p<5x10^{-6}$ from the combined discovery (HapMap and HRC1.1) were followed for replication in three cohorts with available genome-wide data. The SNP-osteocalcin association analysis has been described in the "Osteocalcin Association Analyses" section. A fixed-effect meta-analysis was performed to combine results from these four replication studies. The meta-analysis approach has been described in the "Meta-Analysis" section.

Meta-Analysis of Results from Discovery and Replication Studies

We further combine summary statistics from the discovery studies and replication studies for those SNPs with replication results. The meta-analysis was done by using the fixed-effect sample size weighted meta-analysis implemented in METAL software. These association p-values are considered as "combined association p-values". A successful replication was considered if the combined association p-values were genome-wide significant ($p\leq5x10^{-8}$) and lower than the discovery.

Functional Annotations

We used the FUMA online platform[34] to obtain gene-based, gene-set and tissue-specific annotations. In addition, for all variants, we predicted their function by PolyPhen2.[35] For all variants, we annotated potential regulatory functions of our replicated GWAS SNPs and loci based on experimental epigenetic evidence including DNAse hypersensitive sites, histone modifications, and transcription factor binding sites in human cell lines and tissues from the ENCODE[36,37] and the Epigenetic Roadmap projects. [38] We first constructed haplotype blocks for GWAS lead SNPs and SNPs in high LD ($r2 > 0.8$) with GWAS lead SNPs. We then identified regulatory elements (enhancers and promoters estimated by chromatin state) in the haplotype blocks across 98 healthy human tissues/normal cell lines available in the ENCODE Project and the Epigenetic Roadmap Project using HaploReg4 web browser.[39] GWAS lead SNPs or the SNPs in high LD with GWAS lead SNPs were annotated as to whether they were located within these regulatory elements, including, promoters, flanking active promoters, active promoters, weak promoters, strong and weak enhancers, weak enhancers in transcribed regions,

weak enhancers, active enhancers, active enhancers with weak H3K4me3 and strong H3K27ac and poised enhancers estimated by the ChromHMM approach.[40]

SNP Heritability of Osteocalcin and Genetic Correlations with Energy Metabolic Traits

LD score regression was used to estimate the SNP heritability (h2) of serum osteocalcin and to estimate the genetic correlation (rg) between serum osteocalcin and variety of bone and energy metabolic traits and diseases with publicly available summary GWAS data using LD Hub.[41] The tool is a centralized database of summary-level GWAS results for hundreds of diseases/traits, as well as a web interface that automates the LD score regression analysis pipeline.[42]

Mendelian Randomization Analysis

To determine the causal relation of serum total osteocalcin with the risk of T2D, metabolic relevant disorders, and bone health we selected only GWAS SNPs associated with serum total osteocalcin as instrumental variables from the combined meta-analysis. To estimate the per SNP causal relation between serum total osteocalcin and disease risks, we used the 2-sample MR analytical approach. To increase statistical power, we combined each SNP result into a multi-instrument summary-level MR variable.[43] One of the fundamental assumptions of Mendelian randomization analyses is that there should be no pleiotropy in genetic variants; thus, the genetic variants associated with serum total osteocalcin are exclusively associated with disease risks through only serum total osteocalcin, but no other confounders. To avoid bias of Mendelian randomization analyses from pleiotropic genetic effects, we applied the MR Egger[44] and the Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO).[45] We also performed a bidirectional MR to test for the possibility that BMD may causally affect the levels of total serum osteocalcin. Given several SNPs were associated with both BMD and osteocalcin instead of removing the SNPs we applied Steiger filtering[46] to estimate the true direction of the SNP-trait association.

RESULTS

Genome-Wide Associated Loci and Replication

Sex-combined GWAS meta-analyses were performed on 20,922 European adults. We identified six GWAS loci, including chr1q22 SMG5-BGLAP; chr8q24 TNFRSF11B, chr11q13.1 SSSCA1-AS1, chr11q24.3 FLI1-KCNJ1, chr13q14.11 AKAP11, chr16p13.3 TSC2 (**Table 1**; **Supplementary Figure 1-Manhattan plot**). The QQ plots and λ genome control values of GWAS meta-analyses didn't demonstrate any potential systemic

Table 1 | Conditionally independent SNPs associated with serum total osteocalcin levels ($p<5x10^{-6}$).

							Discovery (N_{max}=20,922)			Replication (N_{max}=9,467)			Combined (N_{max}=30,389)		
Locus	**SNP**	**Closest Gene**	**Annotation**	**EA**	**NEA**	**EAF**	**Effect**	**se**	**P**	**Effect**	**se**	**P**	**Effect**	**se**	**P**
1q22	rs2246476	*SMG5-BGLAP*	intronic	T	C	0.780	0.037	0.004	$2.64x10^{-19}$	0.057	0.016	$3.2x10^{-4}$	0.038	0.004	$3.66x10^{-25}$
			non-coding		C					-0.020	0.019	0.30			
1q22	rs2104404	*TMEM79*	intronic	A		0.201	-0.029	0.004	$6.94x10^{-11}$				-0.030	0.004	$1.83x10^{-12}$
8q24.12	rs10955908	*TNFRSF11B*	intergenic	A	C	0.467	-0.028	0.003	$4.53x10^{-16}$	-0.038	0.013	$2.7x10^{-3}$	-0.030	0.003	$3.73x10^{-22}$
11q13.1	rs1346	*SSSCA1-AS1*	non-coding	A	T	0.812	0.025	0.005	$2.16x10^{-8}$	0.018	0.020	0.36	-0.021	0.004	$1.75x10^{-9}$
11q24.3	rs1787971	*FLI1. KCNJ1*	intergenic	C	G	0.670	-0.020	0.004	$7.73x10^{-8}$	-0.045	0.016	$4.3x10^{-3}$	0.026	0.004	$5.68x10^{-9}$
13q14.11	rs9594738	*TNFSF11*	intergenic	T	C	0.498	0.030	0.003	$5.04x10^{-19}$	0.005	0.015	0.79	0.029	0.003	$8.02x10^{-19}$
16p13.3	rs13337626	*TSC2*	synonymus	T	C	0.916	0.039	0.007	$4.82x10^{-8}$	0.036	0.042	0.40	0.041	0.007	$7.24x10^{-9}$
19q13.41	rs8111274	*FPR3*	intronic	T	G	0.291	-0.021	0.004	$8.25x10^{-8}$	-0.018	0.016	0.28	-0.021	0.004	$1.01x10^{-8}$
1p36.33	rs12758705	*C1orf222*	intronic	A	G	0.269	0.022	0.004	$3.06x10^{-8}$	-0.012	0.017	0.48	0.021	0.004	$1.43x10^{-7}$
			non-coding		G					0.007	0.018	0.71			
1p36.33	rs12081179	*TMEM79*	intronic	A		0.794	-0.022	0.005	$1.47x10^{-6}$				-0.021	0.004	$2.66x10^{-6}$
			non-coding		T					-0.020	0.015	0.19			
1p21.1	rs4908190	*OLFM3*	intronic	A		0.448	-0.017	0.004	$2.26x10^{-6}$				-0.017	0.003	$4.92x10^{7}$
1q41	rs1538742	*RP11-95P13.2*	LincRNA	A	C	0.589	-0.016	0.003	$4.56x10^{-6}$	-0.045	0.016	$4.8x10^{-3}$	-0.017	0.003	$4.11x10^{-7}$
4p14	rs1386625	*RP11-83C7.1*	LincRNA	A	G	0.089	0.029	0.006	$1.42x10^{-6}$	-0.007	0.040	0.85	0.029	0.006	$1.44x10^{-6}$
6q22.31	rs1688630	*CEP85L*	intronic	T	C	0.018	-0.072	0.014	$3.63x10^{-7}$	0.075	0.053	0.16	-0.061	0.014	$9.20x10^{-6}$
6q25.3	rs2181190	*SYNJ2*	intronic	T	C	0.784	-0.020	0.004	$2.90x10^{-6}$	0.010	0.019	0.61	-0.018	0.004	$2.04x10^{-5}$
			non-coding		C					0.036	0.028	0.20			
7p21.1	rs2214919	*AC005062.2*	intronic	T		0.810	-0.023	0.004	$7.65x10^{-8}$				-0.022	0.004	$3.55x10^{-7}$
10q21.2	rs11597844	*ZNF365*	intronic	T	C	0.063	-0.040	0.009	$2.17x10^{-6}$	0.008	0.049	0.88	-0.039	0.008	$3.69x10^{-6}$
11p15.1	rs4325274	*SOX6*	intronic	C	G	0.269	-0.018	0.004	$3.84x10^{-6}$	-0.001	0.017	0.94	-0.016	0.004	$1.63x10^{-5}$
18q21.33	rs884205	*TNFRSF11A*	3utr	A	C	0.268	0.019	0.004	$1.77x10^{-6}$	0.012	0.028	0.96	0.018	0.004	$4.29x10^{-6}$
19q13.41	rs7254413	*FPR3*	intronic	A	G	0.431	-0.017	0.004	$1.90x10^{-6}$	-0.029	0.015	0.05	-0.018	0.003	$1.91x10^{-7}$
20q12	rs6129693	*RNA5SP484*	rRNA	A	C	0.426	-0.017	0.003	$8.68x10^{-7}$	-0.005	0.023	0.85	-0.017	0.003	$8.31x10^{-7}$

EA=effect allele; NEA=non-effect allele; EAF=effect allele frequency; se= standard error; the effect and p-value are for the effect allele

biases (**Supplementary Figure 2**). The most significantly associated SNP, rs2246476, is in the intron region of the SMG5 gene with p-value equal to $2.64x10^{-19}$. The minor allele frequency is 22% in the studied cohorts. We did not observe any differences between the inverse-variance weighted and z-score weighted meta-analysis (Table S3). Therefore, in all post GWAS analysis we used the summary statistics from the IVW meta-analysis.

The GWAS Secondary Signals

To identify independent GWAS signals, we performed a conditional analysis by conditioning on GWAS SNPs. Summary statistics from model 1 and HRC1.1 CEU LD structure were used for the conditional analyses. On Chr1 SMG5 locus (**Supplementary Figure 1**), SNP rs2104404 (**Table 1**), located in the *TMEM79* gene, remained genome-wide significantly associated with serum total osteocalcin after conditioning on the most significant SNP (rs2246476). SNP rs2104404 is not in LD with rs2246476 (R2=0.03, D'=0.56).

Combined Meta-analysis

The top associated SNPs from the HapMap effort and SNPs with $p<5x10^{-6}$ from the total discovery effort were selected for replication. Replication genotyping (including de novo genotyping and genome-wide genotyping) were done in 9,467 Caucasian samples. All GWS loci were successfully replicated in the combined meta-analysis. In addition, one suggestive locus on chr19q13.41 reached GWS in the combined meta-analysis along with 14 suggestive loci (**Table 1**). Overall, seven GWAS loci were identified in the combined meta-analysis. **Supplementary Figure 2** and **Supplementary Figure 3** are the regional plots and forest plots.

Annotation and Functional Role of Associated Variants

We annotated SNPs with discovery p-values ≤5x10-6 from those seven replicated GWAS loci (**Supplementary Table 4**). Except for SNP rs2073618, the other SNPs are intronic, 5'upstream, 3'downstream or intergenic. SNP rs2073618 is a missense variant modifying the third amino acid of OPG protein (encoded by *TNFRSF11B* gene) from Asn to Lys (N3K). SNP rs2073618 was genome-widely associated with serum total osteocalcin and in LD (r2=0.57, D'=0.89) with the top SNP at chr8 (rs10955908). However, the functional impact of this missense SNP is predicted to be "benign" based on the ACMG Gideline.[47]

To annotate potential functions of these non-coding variants and to predict potential functions of these non-coding variants, we overlapped locations of these SNPs with locations of gene regulatory elements, especially on promoters, promoter flanking regions with transcription factor (TF) bindings, enhancers, CTCF binding

sites and TF binding sites (TFBS). The CTCF binding sites represent the activity of insulators, sequences that block the interaction between enhancers and promoters. The average length is 4.3Kb, 1.8Kb, 0.5kb, 0.6kb and 6kb, respectively for promoters, proximal enhancers, distal enhancers, CTCF bindings, and TFBS in human genome. 48 We overlapped SNP locations with these gene regulatory elements observed from human primary osteoblasts. The lead SNPs on chr1 *SMG5-BGLAP*, chr8 -*TNFRSF11B* and chr11 *FLI1* loci are in LD with SNPs located in gene regulatory elements (**Table 2**). rs1346 is located at the promoter flanking TF binding regions of the *SSSCA1-AS1* gene. Moreover, rs2104404, rs10955908, rs611307 and rs11840862 are in LD with SNPs located in the promoter flanking TB bonding region of their corresponding genes. With the exception of rs1246, these regulatory elements were only observed in cells from other lineages but not osteoblasts. (The "Multiple cells" column in **Table 2**). The majority of the associated SNPs were located in the enhancer regions of the mapped genes.

To estimate the confidence of a non-coding variant to be functional, we utilized the RegulomeDB score. It scored variants from 1 (high evidence) to 6 (no or low evidence) based on evidence from eQTL, TF binding, matched TF motif, matched DNase Footprint and DNase peak TF (Table 2). We identified one SNPs (rs6469789) on chr8 TNFRSF11B locus scored as "1d" (evidences from eQTL + TF binding + any motif + DNase peak). In addition, 7 SNPs on chr1 SMG5-BGLAP locus scored as "1f" (evidences from eQTL + TF binding + matched TF motif). Among them, only rs2246476 was a lead SNP. The SNPs scored as "1d" or "1f" are likely to affect binding and linked to expression of a gene target.

Gene-based association Analyses

The gene-based association test implemented in MAGMA 49 using the FUMA platform was performed using summary statistics from discovery GWAS meta-analyses. All available SNPs were mapped to 18,793 protein coding genes. Genome-wide significance was found for *TNFRSF11B, SMG5, CCT3, TMRM79, PMF1-BGLAP* and *PAQR6* genes with p-values ≤ 2.6x10-6 (=0.05/18793) after Bonferroni correction (**Supplementary Figure 5**). *TSC2* and *FPR3* were also strongly associated.

Chromatin 3D Interactions

Hi-C looping structure (chromatin 3D interactions) was used to find interactions between SNPs located regions and potential promoter regions of protein coding genes. We only considered looping structures that overlapped with estimated enhancers and promoters, so that chromatin interactions are always between enhancer regions and promoter regions. We combined chromatin interactions as well as gene regulatory element information (enhancers and promoters) from multiple tissues together; thus,

Table 2 | Annotation of lead SNPs and SNPs in LD in osteoblast and other type of cells.

CHR	Lead SNP	SNP in LD	(r^2)	Position	RegulomeDB	Feature Type	Osteoblasts	Other cell tissues	annotation	Genes
1	rs2246476		1.00	156271910	**1f**	Enhancer		x	intronic	SMG5
1		rs2253358	0.81	156258269	**1f**	Enhancer		x	intronic	SMG5
1		rs2985711	0.80	156312433	7	CTCF Binding Site	x	x	intronic	CCT3
1		rs2495269	0.80	156312858	6	CTCF Binding Site	x	x	intronic	CCT3
1	rs2277872		0.98	156276937	6	Promoter flanking with TF		x	intronic	SMG5
1	rs2104404	rs2104404	0.98	156287122	5	CTCF Binding Site			intronic	TMEM79
1		rs1543294	0.84	156240066	**1f**	Enhancer	x	x	3'-UTR	PMF1-BGLAP
1		rs12090516	0.84	156240766	4	Enhancer		x	intronic	RP11-54H19.8
1		rs2287025	0.84	156260350	7	Enhancer		x	intronic	SMG5
1		rs2287024	0.84	156260415	**1f**	Enhancer		x	intronic	SMG5
1		rs759328	0.84	156261475	**1f**	Enhancer		x	intronic	SMG5
1		rs4661036	0.88	156263688	6	Enhancer	x	x	intronic	SMG5
1		rs35478936	0.85	156335371	**1f**	Enhancer		x	intronic	CCT3
1		rs4661037	0.85	156268688	**1f**	Enhancer	x	x	intronic	SMG5
1		rs16837272	0.81	156271044	7	Enhancer		x	intronic	SMG5
1		rs4661038	1.00	156288712	5	CTCF Binding Site	x	x	intronic	TMEM79
8	rs10955908			118892318	5			x		31kb 3' of TNFRSF11B
8		rs6469788	0.80	118940511	7	Promoter flanking with TF		x	intronic	TNFRSF11B
8		rs6469789	0.81	118948422	**1d**	Promoter flanking with TF		x	intronic	TNFRSF11B
8		rs4407910	1.00	118904878	7	Enhancer	x	x		19kb 3' of TNFRSF11B
8		rs13439134	0.99	118906629	5	Enhancer		x		17kb 3' of TNFRSF11B
8		rs13277230	0.99	118910754	5	Enhancer	x	x		13kb 3' of TNFRSF11B
8		rs4355801	0.99	118911634	5	Enhancer	x	x		12kb 3' of TNFRSF11B
8		rs4319131	0.80	118935412	5	Enhancer	x	x	intronic	TNFRSF11B
8		rs7463176	0.81	118945386	7	Enhancer		x	intronic	TNFRSF11B
8		rs11573829	0.81	118947384	5	Enhancer	x	x	intronic	TNFRSF11B

Table 2 | Annotation of lead SNPs and SNPs in LD in osteoblast and other type of cells. (*continued*)

CHR	Lead SNP	SNP in LD	(r^2)	Position	RegulomeDB	Feature Type	Osteoblasts	Other cell tissues	annotation	Genes
8		rs4242592	0.80	118956736	4	Enhancer		x		4.5kb 5' of TNFRSF11B
8		rs7006553	0.80	118958540	7	Enhancer		x		6.3kb 5' of TNFRSF11B
8		rs2062375	0.81	118965553	6	Enhancer		x		11kb 5' of U6
8		rs10101385	0.99	118911611	6	CTCF Binding Site		x		12kb 3' of TNFRSF11B
8		rs13250753	1.00	118894453	7	CTCF Binding Site		x		29kb 3' of TNFRSF11B
8		rs6415470	0.81	118942872	7	CTCF Binding Site		x	intronic	TNFRSF11B
8		rs10505348	0.80	118960457	7	CTCF Binding Site		x		8.3kb 5' of TNFRSF11B
11	rs1346	rs1346		65569780	4	Promoter flanking with TF	x	x		SSSCA1-AS1
11	rs611307	rs611307		128799925	5	Enhancer		x	intronic	FLI1
11		rs1787971	0.97	128813668	7	Enhancer		x		401bp 3' of FLI1
11		rs608764	0.95	128790125	5	Promoter flanking with TF	x	x	intronic	FLI1
11		rs549809	1.00	128815422	4	Promoter flanking with TF	e	x		2.2kb 3' of FLI1
11		rs550621	0.80	128815467	**3a**	Promoter flanking with TF	e	x		2.2kb 3' of FLI1
11		rs680952	0.96	128794013	6	Enhancer		x	intronic	FLI1
11		rs648193	0.96	128797907	5	Enhancer		x	intronic	FLI1
11		rs611307	0.97	128799925	5	Enhancer		x	intronic	FLI1
11		rs579355	0.97	128806265	7	Enhancer		x	intronic	FLI1
11		rs582825	1.00	128809518	4	Enhancer		x	intronic	FLI1
11		rs619255	1.00	128809555	5	Enhancer		x	intronic	FLI1
11		rs682695	1.00	128812565	**3a**	Enhancer		x	3'-UTR	FLI1
11		rs522331	1.00	128814724	6	Enhancer		x		1.5kb 3' of FLI1
11		rs681296	1.00	128816368	5	Enhancer	x	x		3.1kb 3' of FLI1
11		rs496501	1.00	128817050	5	Enhancer		x		3.8kb 3' of FLI1
11		rs500081	0.80	128817410	7	CTCF Binding Site		x		4.1kb 3' of FLI1
11		rs654788	1.00	128817755	5	CTCF Binding Site		x		4.5kb 3' of FLI1

Table 2 | Annotation of lead SNPs and SNPs in LD in osteoblast and other type of cells. (*continued*)

CHR	Lead SNP	SNP in LD	(r^2)	Position	RegulomeDB	Feature Type	Osteoblasts	Other cell tissues	annotation	Genes
11		rs654344	1.00	128817851	5	CTCF Binding Site		x		4.6kb 3' of FLI1
11		rs617954	1.00	128817901	7	CTCF Binding Site		x		4.6kb 3' of FLI1
11		rs504760	0.80	128817940	6	CTCF Binding Site		x		4.7kb 3' of FLI1
12	rs11840862	rs11840862		42382327	5	Enchancer		x		59kb 3' of AKAP11
13		rs11840862	0.40	42378009	4	Enhancer	x	x		54kb 3' of AKAP11
13		rs9533090	0.40	42377313	4	Promoter flanking with TF		x		54kb 3' of AKAP11
16	rs13337626	rs13337626	1.00	2075833	5	Enchancer			synonymous	TSC2
16		rs1800715	0.95	2075936	5	Enhancer		x	intronic	TSC2
19	rs8111274	rs8111274		51801340	5	Enchancer			intronic	FPR3

results are not tissue-type or cell-type specific. As showed on the circus plot (**Supplementary Figure 6A**) of chr1 *SMG5-BGLAP* locus, the chromatin interactions (colored orange) were found between top associated SNPs and several genes in the regions. eQTLs between top associated SNPs and genes were highlighted in green. Genes highlighted in red, including *SMG5*, *BGLAP* among others, showed both evidences from chromatin interactions and eQTLs. On chr 8 *TNFRSF11B*, *COLE10*, *MAL2*, *NOV* and *SMAD12* were all marked by significant chromatin interactions and eQTLs (**Supplementary Figure 6B**). Next, on chr11q24.3 both *KCNJ1* and *FLJ1* can be considered possible candidate genes, whereas on chr11q13.1 the list of genes marked by both chromatin interaction and eQTLs is large (**Supplementary Figure 6c** and **Supplementary Figure 6D**). The chr 13 locus was highlighted by AKAP11(**Supplementary Figure 6E**). Finally, on chr16 and chr19 (**Supplementary Figure 6F** and **Supplementary Figure 6g**, respectively), *TSC2* and *FPPR3* are likely to be the candidate genes with support from chromatin interactions and eQTLs.

Heritability and Genetic Correlations

The estimated SNP heritability (h2) of serum total osteocalcin is 9% (95%CI: 4-12%). We found negative genetic correlations between serum total osteocalcin and triglycerides, waist-to-hip ratio, type 2 diabetes and femoral neck BMD ($p<0.05$). Positive genetic correlation was also found for height (genetic correlation=0.15) (**Figure 1**). Since BMI was adjusted in our osteocalcin GWAS analyses, the genetic correlations with quantitative BMI relevant phenotypes may not be estimated accurately.

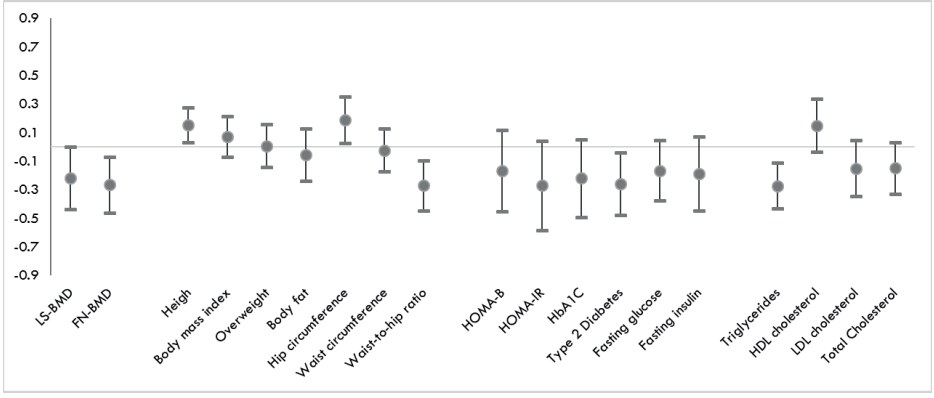


Figure 1 | Genetic correlation between osteocalcin and variety of related traits. LS-BMD=Lumbar spine bone mineral density; FN-BMD= femoral neck bone mineral density. HOMA-B= Homeostatic model assessment of β-cell function idex; HOMA-IR= Homeostatic model assessment of insulin resistance index; HDL= high-density lipoproteins; LDL= low-density lipoproteins.

Mendelian Randomization Analyses

SNPs from seven replicated GWAS loci were selected as instrumental variables for total serum osteocalcin. We did not detect any pleiotropic biases in our MR analysis (Egger-intercept). We did not find any causal effect of genetically determined osteocalcin on type 2 diabetes and other glycemic traits with the exception of HOMA-IR (**Table 3**). We did not look into obesity relevant phenotypes (BMI, waist-to-hip ration, obesity) since BMI was adjusted in our osteocalcin GWAS analyses; therefore, the analyses may not be accurately.

As a positive control, we also performed Mendelian randomization analyses on bone relevant phenotypes. We found a causal effect of serum total osteocalcin on lower femoral-neck BMD (beta=-1.32; p-value=$1.4x10^{-6}$) (**Table 4**).

Table 3 | Causal effect of serum total osteocalcin on different bone and metabolic traits.

Outcome	*IVW*		*Weighted median*		*MR-PRESSO*			*Egger intercept*
	effect (95%CI)	**P**	**Effect (95%CI)**	**P**	**global P**	**Effect (95%CI)**	**P**	**P**
Femoral neck BMD	-0.685 (-1.239, -0.130)	0.016	-0.707 (-1.111, -0.304)	0.001	<0.001	-0.715 (-1.173, -0.257)	0.028	0.51
Lumbar spine BMD	-1.056 (-1.948, -0.163)	0.020	-0.590 (-1.106, -0.073)	0.026	<0.001	-0.616 (-1.087, -0.145)	0.083	0.94
Type 2 Diabetes	-0.395 (-1.046, 0.256)	0.234	-0.216 (-0.651, 0.234)	0.331	0.001	-0.370 (-0.729, 0.010)	0.100	0.19
Fasting glucose	-0.048 (-0.140, 0.044)	0.303	-0.017 (-0.136, 0.102)	0.781	0.583	NA	NA	0.67
Fasting insulin	-0.042 (-0.136, 0.052)	0.384	-0.062 (-0.190, 0.065)	0.775	0.421	NA	NA	0.51
Homa-B	-0.083 (-0.203, 0.038)	0.180	-0.181 (-0.232, 0.040)	0.012	0.159	NA	NA	0.02
Homa-IR	-0.128 (-0.250, -0.006)	0.041	-0.183 (-0.341, -0.026)	0.022	0.818	NA	NA	0.22

global p= test for horizontal pleiotropy; MR-Presso beta (95%CI) = corrected effect estimate for horizontal pleiotropy via outlier removal; NA= not applicable. If the global test is not significant (P<0.05) corrected effect is not calculated as there are no outliers

Table 4 | Causal effect of BMD on osteocalcin.

Exposure	*IVW*		*Weighted median*		*MR-PRESSO*			*Egger intercept*
	beta (95%CI)	**P**	**beta (95%CI)**	**P**	**global P**	**beta (95%CI)**	**P**	**P**
Femoral neck BMD	-0.065 (-0.111, -0.019)	0.005	-0.035 (-0.069, -0.000)	0.048	<0.001	-0.042 (-0.069 -0.015)	0.004	0.85
Lumbar spine BMD	-0.056 (-0.097, -0.015)	0.008	-0.028 (-0.062, 0.005)	0.09	<0.001	-0.018 (-0.041, -0.005)	0.14	0.01

global p= test for horizontal pleiotropy; MR-Presso beta (95%CI) = corrected effect estimates for horizontal pleiotropy via outlier removal; NA= not applicable. If the global test is not significant (P<0.05) corrected effect is not calculated as there are no outlier

DISCUSSION

In the current GWAS study, we successfully replicated seven GWAS loci associated with total serum osteocalcin. Several of the SNPs were located within or near genes implicated in relevant bone pathways such as *TNFRSF11B* and *AKAP11*. Total serum osteocalcin had modest heritability (~9%) and weak evidence of shared genetic etiology with femoral neck BMD, type 2 diabetes, triglycerides, waist-to-hip ratio and height. Nonetheless, none of these relationships were causal with the exception of BMD.

The strongest associations arose from the 1q22 locus which harbors the well-known osteocalcin gene, *BGLAP*, but also several other genes such as *SMG5*, *TMEM79* and *CCT3*. The strongest association (rs2246476) mapped to the *SMG5* gene. This SNP or its proxies (r^2>0.8) have not been reported to be associated with any other traits before. Although *SMG5* have been associated with variety of traits such as mortality, HbA1c, red cell distribution, and adiposity the lead SNPs in these associations were not in LD with our lead SNP(r^2<0.2). This SNP is highly likely to affect the binding and expression of *SMG5* (regulomeDB score of 1f [eQTL + TF binding/DNase peak]). In addition, the variant is located within the enhancer region of SMG5 but only in the liver. Additional secondary signal in this locus (rs2277872) overlap with the TF Promoter flanking region of *SMG5* in blood cells and the enhancer region in osteoblasts. The biological function of *SMG5* in not well understood. What is known so far is that it plays important role in nonsense-mediated mRNA decay; process relevant to reduce errors during gene expression.[50] The gene is expressed across various tissues such as testis, blood, and muscle among others. In the proximity of *SMG5* is the *BGLAP* or the so called "osteocalcin gene". *BGLAP* encodes 4 exons from which the first three produce immature proteins, whereas exon 4 produces mature one. [51] In osteoblasts,

the expression of osteocalcin is incited by the Runx2 (Cbfa1) transcription factor; without it the osteoblasts will don't differentiate and produce bone[52] and osteocalcin will not be produced as well. On the other hand, BGLAP expression is inhibited by the transforming growth factor-β (TGF-β) in an autocrine fashion. The *BGLAP* promotor is sensitive to vitamin D, alcohol intake and corticosteroids, thus, they may influence the levels of total serum osteocalcin. The lead SNPs in this locus are also in LD with SNPs mapping near *TMEM79* and *CCT3*; thus, any of these genes can be a potential candidate gene. Nevertheless, *SMG5* and *BGLAP* were highlighted by both chromatin interactions and eQTLs; making them stronger candidate genes in this locus.

Several SNPs mapped to genes involved in the nuclear factor-κB ligand (RANKL/ RANK /osteoprotegerin (OPG)) signaling pathway such as *TNFRSF11B* and *AKAP11*. *TNFRSF11B* encodes the OPG protein which is primarily secreted from the osteoblasts and it is essential for osteoclast development and function. OPG binds to RANKL, and prevents it from activating its cognate receptor RANK, which is vital for osteoclast differentiation, activation and survival.[53] Next, the *AKAP11* gene (is located ~185 kb upstream of RANKL) encodes a group of A-kinase anchor proteins (AKAP), involved in intracellular signaling events by linking cAMP to other pathways.[54,55] In mice, AKAP11 is continuously expressed during osteoblast maturation.[56] Akap11 knockout cells, generated using CRISPR/Cas9 in mouse pre-osteoblastic MC3T3-E1 cells, presented significant reduction of calcium mineral deposition and decreased alkaline phosphatase (ALP) activity since differentiation phase (Day7). Moreover, the expression of the major structural protein genes such as OC, IBSP and SPP1, was reduced since mineralization phase (Day16).[56] *TNFRSF11B* and *AKAP11* were mapped by both chromatin interaction and eQTLs. Signals also arose from the 11q24.3 locus that have been recently associated with heel BMD[57] which mapped near *FLI1* that have also been linked to the NF-kB Signaling; however, the exact mechanism of action of this gene on bone are still unknown. Overall, these genes play a key role in bone metabolism, thus, can affect the serum osteocalcin levels as well.

Next, we identified two loci that have not been associated with any bone traits harboring the *TSC2* and *FPR3* genes. Nevertheless, these genes have shown links to relevant bone pathways. For instance, *TSC2* been related with the mTOR signaling pathway[58] implicated in osteoblast differentiation and function stimulated by several bone anabolic ligands such as Wnt, igf and bmp.[59,60,61] The role of mTOR signaling in osteoclastogenesis is still controversial as inactivation of mTORC1 by deletion of Raptor can enhance, whereas hyperactivation of mTORC1 by deleting Tsc1 in osteoclast precursors can impair osteoclastogenesis.[62] *FPR3* on the other hand, has been proposed to promote osteoblast differentiation via the N-Formyl Peptide Receptor 1-mediated Signaling Pathway in Human Mesenchymal Stem Cells from Bone Marrow GPCRs (G protein coupled receptor) in bone.[63] G protein signaling also has a key role in

bone development and remodeling by influencing the GPCR-G, Wnt, calcium and PTH signaling pathways. In addition, the G-protein-coupled receptor (GPCR) 48 have been related with significant downregulation of the expression levels of osteocalcin and collagen.[64] Interestingly, GPCR represents the largest family of proteins that is currently targeted by approve drugs. [65] The exact role of these genes in the underlaying pathways is still unknown and warrants further investigation.

In the past decade there has been increasing recognition of the intimate relationship between bone and energy metabolism. Initially it was shown thar energy metabolism influence bone remodeling through the effects of leptin, serotonin and the sympathetic tone [reviewed elsewhere [66]]. Therefore, it was postulated that bone may also provide a feedback loop and affect energy metabolism. An early experimental animal study showed that osteocalcin-deficient mice were glucose intolerant, insulin resistant and fat started.[5] Similarly, observational studies have shown inverse association between osteocalcin, adiposity and diabetes.[67,68,69,70] We did find a negative genetic correlation between osteocalcin and type 2 diabetes. However, our MR analysis, did not show any causal effect of genetically determined total serum osteocalcin on type 2 diabetes; indicating that osteocalcin and type 2 diabetes may share common pathways and the observed associations to be simple due to pleiotropy. In addition, osteocalcin and BMD showed a bidirectional association driven by the SNPs mapping to the RANK/RANK/OPG pathway; establishing the role of osteocalcin as bone turnover marker where as evidence of a causal effect cannot be established. Overall, the MR results need to be interpreted with caution as the variance explained of osteocalcin was low; thus, providing low power to detect any true causal associations.

This study has several limitations that need to be highlighted. First, the majority of the studies were imputed to older imputation panels (HapMap) and only one utilized newer and denser panel (HRC1.1); thus, we might have missed some variants associated with total serum osteocalcin not present in the majority of the studies. Second, the osteocalcin assays were different between studies which may have led to lower power to detect true-positive associations. Finally, we were not able to examine the carboxylated and non-carboxylated forms of osteocalcin separately as the majority of the cohorts estimated only the total serum osteocalcin.

Total serum osteocalcin is a complex, heritable trait implicated in relevant bone mechanisms. We identified seven loci robustly associated with total serum osteocalcin. MR analysis showed no causal link with metabolic and glycemic traits, but did proved evidence for bidirectional association with BMD, highlighting the essential role of osteocalcin in bone biology. The exact role of osteocalcin in energy metabolism in humans is yet to be full elucidate till then our results do not support any causal association.

REFERENCES

1. Hauschka P V., Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: Vitamin K-dependent proteins in bone. *Physiol Rev*. 1989;69(3):990-1047. doi:10.1152/physrev.1989.69.3.990
2. CALVO MS, EYRE DR, GUNDBERG CM. Molecular Basis and Clinical Application of Biological Markers of Bone Turnover*. *Endocr Rev*. 1996;17(4):333-368. doi:10.1210/edrv-17-4-333
3. Lian JB, Gundberg CM. Osteocalcin. Biochemical considerations and clinical applications. *Clin Orthop Relat Res*. 1988;(226):267-291. doi:10.1097/00003086-198801000-00036
4. Gundberg CM, Weinstein RS. Multiple immunoreactive forms of osteocalcin in uremic serum. *J Clin Invest*. 1986;77(6):1762-1767. doi:10.1172/JCI112499
5. Moser SC, van der Eerden BCJ. Osteocalcin—A Versatile Bone-Derived Hormone. *Front Endocrinol (Lausanne)*. 2019;9:794. doi:10.3389/fendo.2018.00794
6. Oury F, Sumara G, Sumara O, et al. Endocrine Regulation of Male Fertility by the Skeleton. *Cell*. 2011;144(5):796-809. doi:10.1016/j.cell.2011.02.004
7. Mera P, Laue K, Ferron M, et al. Osteocalcin Signaling in Myofibers Is Necessary and Sufficient for Optimum Adaptation to Exercise. *Cell Metab*. 2016;23(6):1078-1092. doi:10.1016/j.cmet.2016.05.004
8. Oury F, Khrimian L, Denny CA, et al. XMaternal and offspring pools of osteocalcin influence brain development and functions. *Cell*. 2013;155(1):228. doi:10.1016/j.cell.2013.08.042
9. Khrimian L, Obri A, Ramos-Brossier M, et al. Gpr158 mediates osteocalcin's regulation of cognition. *J Exp Med*. 2017;214(10):2859-2873. doi:10.1084/jem.20171320
10. Hinoi E, Gao N, Jung DY, et al. The sympathetic tone mediates leptin's inhibition of insulin secretion by modulating osteocalcin bioactivity. *J Cell Biol*. 2008;183(7):1235-1242. doi:10.1083/jcb.200809113
11. Hinoi E, Gao N, Jung DY, et al. An osteoblast-dependent mechanism contributes to the leptin regulation of insulin secretion. *Ann N Y Acad Sci*. 2009;1173(SUPPL. 1):E20-30. doi:10.1111/j.1749-6632.2009.05061.x
12. Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci*. 2008;105(13):5266-5270. doi:10.1073/pnas.0711119105
13. Díaz-López A, Bulló M, Juanola-Falgarona M, et al. Reduced serum concentrations of carboxylated and undercarboxylated osteocalcin are associated with risk of developing type 2 diabetes mellitus in a high cardiovascular risk population: A nested case-control study. *J Clin Endocrinol Metab*. 2013;98(11):4524-4531. doi:10.1210/jc.2013-2472
14. Kindblom JM, Ohlsson C, Ljunggren Ö, et al. Plasma Osteocalcin Is Inversely Related to Fat Mass and Plasma Glucose in Elderly Swedish Men. *J Bone Miner Res*. 2009;24(5):785-791. doi:10.1359/jbmr.081234
15. Liatis S, Sfikakis PP, Tsiakou A, et al. Baseline osteocalcin levels and incident diabetes in a 3-year prospective study of high-risk individuals. *Diabetes Metab*. 2014;40(3):198-203. doi:10.1016/j.diabet.2014.01.001
16. Hong S, Koo J, Hwang JK, et al. Changes in Serum Osteocalcin are Not Associated with Changes in Glucose or Insulin for Osteoporotic Patients Treated with Bisphosphonate. *J Bone Metab*. 2013;20(1):37. doi:10.11005/jbm.2013.20.1.37
17. Storm D, Porter REES, Musgrave K, et al. Calcium Supplementation Prevents Seasonal Bone Loss and Changes in Biochemical Markers of Bone Turnover in Elderly New England Wom-

en: A Randomized Placebo-Controlled Trial 1 . *J Clin Endocrinol Metab*. 1998;83(11):3817-3825. doi:10.1210/jcem.83.11.5289

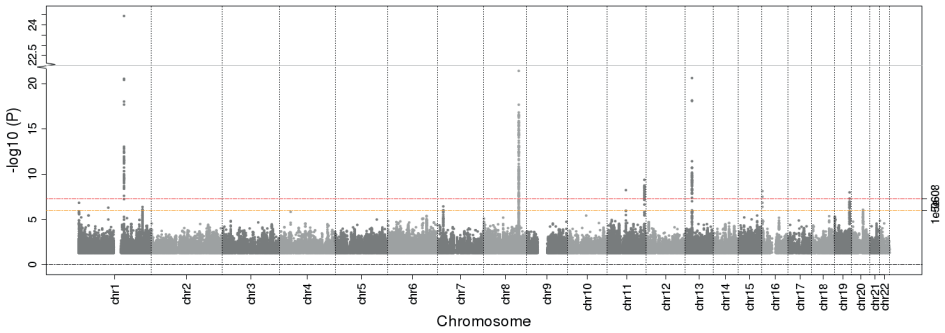
18. Woitge HW, Knothe A, Witte K, et al. Circannual rhythms and interactions of vitamin D metabolites, parathyroid hormone, and biochemical markers of skeletal homeostasis: A prospective study. *J Bone Miner Res*. 2000;15(12):2443-2450. doi:10.1359/jbmr.2000.15.12.2443
19. Vanderschueren D, Gevers G, Raymaekers G, Devos P, Dequeker J. Sex- and age-related changes in bone and serum osteocalcin. *Calcif Tissue Int*. 1990;46(3):179-182. doi:10.1007/bf02555041
20. Johansen JS, Giwercman A, Hartwell D, et al. Serum Bone Gla-Protein as a Marker of Bone Growth in Children and Adolescents: Correlation With Age, Height, Serum Insulin-Like Growth Factor I, and Serum Testosterone. *J Clin Endocrinol Metab*. 1988;67(2):273-278. doi:10.1210/jcem-67-2-273
21. Yasui T, Uemura H, Tomita J, et al. Association of serum undercarboxylated osteocalcim with serum estradiol in pre-, peri- and early post-menopausal women. *J Endocrinol Invest*. 2006;29(10):913-918. doi:10.1007/BF03349196
22. Mitchell BD, Cole SA, Bauer RL, et al. Genes Influencing Variation in Serum Osteocalcin Concentrations Are Linked to Markers on Chromosomes 16q and 20q 1 . *J Clin Endocrinol Metab*. 2000;85(4):1362-1366. doi:10.1210/jcem.85.4.6571
23. Dohi Y, Iki M, Ohgushi H, et al. A novel polymorphism in the promoter region for the human osteocalcin gene: The possibility of a correlation with bone mineral density in postmenopausal Japanese women. *J Bone Miner Res*. 1998;13(10):1633-1639. doi:10.1359/jbmr.1998.13.10.1633
24. Gustavsson A, Nordström P, Lorentzon R, Lerner UH, Lorentzon M. Osteocalcin Gene Polymorphism is Related to Bone Density in Healthy Adolescent Females. *Osteoporos Int*. 2000;11(10):847-851. doi:10.1007/s001980070043
25. Jiang DK, Xu FH, Liu MY, et al. No evidence of association of the osteocalcin gene HindIII polymorphism with bone mineral density in Chinese women. *J Musculoskelet Neuronal Interact*. 7(2):149-154. http://www.ncbi.nlm.nih.gov/pubmed/17627084. Accessed April 1, 2020.
26. Marchini J, Howie B, Myers S, McVean G, Donnelly P. A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat Genet*. 2007;39(7):906-913. doi:10.1038/ng2088
27. Howie BN, Donnelly P, Marchini J. A Flexible and Accurate Genotype Imputation Method for the Next Generation of Genome-Wide Association Studies. Schork NJ, ed. *PLoS Genet*. 2009;5(6):e1000529. doi:10.1371/journal.pgen.1000529
28. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: Using sequence and genotype data to estimate haplotypes and unobserved genotypes. *Genet Epidemiol*. 2010;34(8):816-834. doi:10.1002/gepi.20533
29. Howie B, Fuchsberger C, Stephens M, Marchini J, Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet*. 2012;44(8):955-959. doi:10.1038/ng.2354
30. Consortium the HR, McCarthy S, Das S, et al. A reference panel of 64,976 haplotypes for genotype imputation. *Nat Genet*. 2016;48(10):1279-1283. doi:10.1038/ng.3643

31. Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: regional visualization of genome-wide association scan results. *Bioinformatics*. 2010;26(18):2336-2337. doi:10.1093/bioinformatics/btq419
32. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. *Am J Hum Genet*. 2011;88(1):76-82. doi:10.1016/j.ajhg.2010.11.011
33. van Wijngaarden JP, Dhonukshe-Rutten RA, van Schoor NM, et al. Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12and folic acid on fracture incidence. *BMC Geriatr*. 2011;11(1):80. doi:10.1186/1471-2318-11-80
34. Zheng J, Erzurumluoglu AM, Elsworth BL, et al. LD Hub: a centralized database and web interface to perform LD score regression that maximizes the potential of summary level GWAS data for SNP heritability and genetic correlation analysis. *Bioinformatics*. 2017;33(2):272-279. doi:10.1093/bioinformatics/btw613
35. Bulik-Sullivan BK, Loh P-R, Finucane HK, et al. LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat Genet*. 2015;47(3):291-295. doi:10.1038/ng.3211
36. Burgess S, Bowden J, Fall T, Ingelsson E, Thompson SG. Sensitivity analyses for robust causal inference from mendelian randomization analyses with multiple genetic variants. *Epidemiology*. 2017;28(1):30-42. doi:10.1097/EDE.0000000000000559
37. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol*. 2015;(June):512-525. doi:10.1093/ije/dyv080
38. Verbanck M, Chen CY, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat Genet*. 2018;50(5):693-698. doi:10.1038/s41588-018-0099-7
39. Hemani G, Tilling K, Davey Smith G. Orienting the causal relationship between imprecisely measured traits using GWAS summary data. Li J, ed. *PLOS Genet*. 2017;13(11):e1007081. doi:10.1371/journal.pgen.1007081
40. Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. *Nat Commun*. 2017;8(1):1826. doi:10.1038/s41467-017-01261-5
41. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet*. 2013;0 7(SUPPL.76):Unit7.20. doi:10.1002/0471142905.hg0720s76
42. Neph S, Vierstra J, Stergachis AB, et al. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature*. 2012;489(7414):83-90. doi:10.1038/nature11212
43. Gerstein MB, Kundaje A, Hariharan M, et al. Architecture of the human regulatory network derived from ENCODE data. *Nature*. 2012;489(7414):91-100. doi:10.1038/nature11245
44. Roadmap Epigenomics Consortium, Kundaje A, Meuleman W, et al. Integrative analysis of 111 reference human epigenomes. *Nature*. 2015;518(7539):317-329. doi:10.1038/nature14248
45. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res*. 2012;40(Database issue):D930-4. doi:10.1093/nar/gkr917
46. Ernst J, Kellis M. ChromHMM: Automating chromatin-state discovery and characterization. *Nat Methods*. 2012;9(3):215-216. doi:10.1038/nmeth.1906

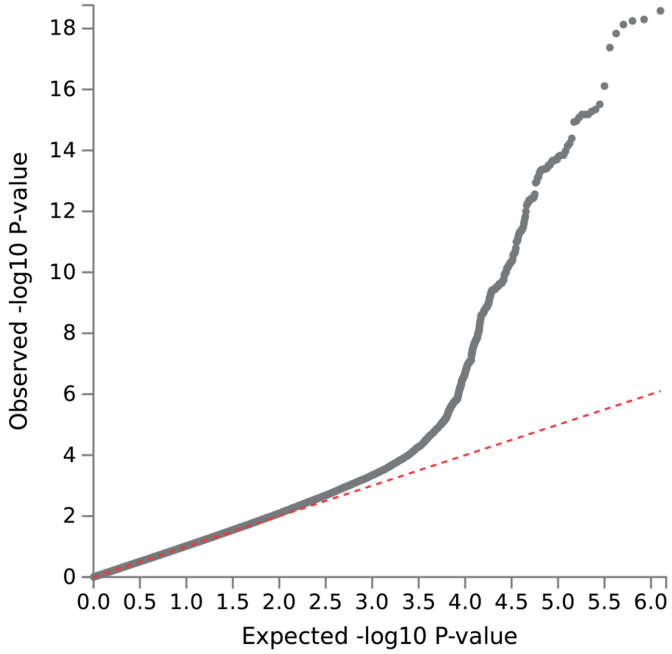
47. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424. doi:10.1038/gim.2015.30
48. Zerbino DR, Wilder SP, Johnson N, Juettemann T, Flicek PR. The Ensembl Regulatory Build. *Genome Biol*. 2015;16(1):56. doi:10.1186/s13059-015-0621-5
49. de Leeuw CA, Mooij JM, Heskes T, Posthuma D. MAGMA: Generalized Gene-Set Analysis of GWAS Data. Tang H, ed. *PLOS Comput Biol*. 2015;11(4):e1004219. doi:10.1371/journal.pcbi.1004219
50. Nickless A, Bailis JM, You Z. Control of gene expression through the nonsense-mediated RNA decay pathway. *Cell Biosci*. 2017;7(1). doi:10.1186/s13578-017-0153-7
51. McKee MD, Cole WG. Bone Matrix and Mineralization. In: *Pediatric Bone*. Elsevier Inc.; 2012:9-37. doi:10.1016/B978-0-12-382040-2.10002-4
52. Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997;89(5):755-764. doi:10.1016/S0092-8674(00)80258-5
53. Leibbrandt A, Penninger JM. RANK–RANKL Signaling. In: *Encyclopedia of Cancer*. Springer Berlin Heidelberg; 2008:2537-2540. doi:10.1007/978-3-540-47648-1_4945
54. Carnegie GK, Means CK, Scott JD. A-kinase anchoring proteins: From protein complexes to physiology and disease. *IUBMB Life*. 2009;61(4):394-406. doi:10.1002/iub.168
55. AKAP11 A-kinase anchoring protein 11 [Homo sapiens (human)] - Gene - NCBI. https://www.ncbi.nlm.nih.gov/gene/11215. Accessed April 1, 2020.
56. Cheng VKF, Li GHY, Tan KCB Lu W, Cheung C. AKAP11 is a positive regulator of osteoblast extracellular matrix formation and mineralization. *46th Annu Meet Eur Calcif Tissue Soc (ECTS 2019)*.
57. Li Y, Corradetti MN, Inoki K, Guan KL. TSC2: Filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci*. 2004;29(1):32-38. doi:10.1016/j.tibs.2003.11.007
58. Inoki K, Ouyang H, Zhu T, et al. TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth. *Cell*. 2006;126(5):955-968. doi:10.1016/j.cell.2006.06.055
59. Xian L, Wu X, Pang L, et al. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nat Med*. 2012;18(7):1095-1101. doi:10.1038/nm.2793
60. Karner CM, Lee S-Y, Long F. Bmp Induces Osteoblast Differentiation through both Smad4 and mTORC1 Signaling. *Mol Cell Biol*. 2017;37(4). doi:10.1128/mcb.00253-16
61. Zhang Y, Xu S, Li K, et al. mTORC1 Inhibits NF-κB/NFATc1 Signaling and Prevents Osteoclast Precursor Differentiation, In Vitro and In Mice. *J Bone Miner Res*. 2017;32(9):1829-1840. doi:10.1002/jbmr.3172
62. Shin MK, Jang YH, Yoo HJ, et al. N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP) promotes osteoblast differentiation via the N-formyl peptide receptor 1-mediated signaling pathway in human mesenchymal stem cells from bone marrow. *J Biol Chem*. 2011;286(19):17133-17143. doi:10.1074/jbc.M110.197772
63. Luo J, Zhou W, Zhou X, et al. Regulation of bone formation and remodeling by G-protein-coupled receptor 48. *Development*. 2009;136(16):2747-2756. doi:10.1242/dev.033571
64. Sriram K, Insel PA. G protein-coupled receptors as targets for approved drugs: How many targets and how many drugs? In: *Molecular Pharmacology*. Vol 93. American Society for Pharmacology and Experimental Therapy; 2018:251-258. doi:10.1124/mol.117.111062

65. Confavreux CB. Bone: from a reservoir of minerals to a regulator of energy metabolism. *Kidney Int Suppl*. 2011;79(121):S14-S19. doi:10.1038/ki.2011.25
66. Takashi Y, Ishizu M, Mori H, et al. Circulating osteocalcin as a bone-derived hormone is inversely correlated with body fat in patients with type 1 diabetes. Mogi M, ed. *PLoS One*. 2019;14(5):e0216416. doi:10.1371/journal.pone.0216416
67. Polgreen LE, Jacobs DR, Nathan BM, et al. Association of osteocalcin with obesity, insulin resistance, and cardiovascular risk factors in young adults. *Obesity (Silver Spring)*. 2012;20(11):2194-2201. doi:10.1038/oby.2012.108
68. Kanazawa I, Yamaguchi T, Yamauchi M, et al. Serum undercarboxylated osteocalcin was inversely associated with plasma glucose level and fat mass in type 2 diabetes mellitus. *Osteoporos Int*. 2011;22(1):187-194. doi:10.1007/s00198-010-1184-7
69. Ngarmukos C, Chailurkit L, Chanprasertyothin S, Hengprasith B, Sritara P, Ongphiphadhanakul B. A reduced serum level of total osteocalcin in men predicts the development of diabetes in a long-term follow-up cohort. *Clin Endocrinol (Oxf)*. 2012;77(1):42-46. doi:10.1111/j.1365-2265.2011.04215.x

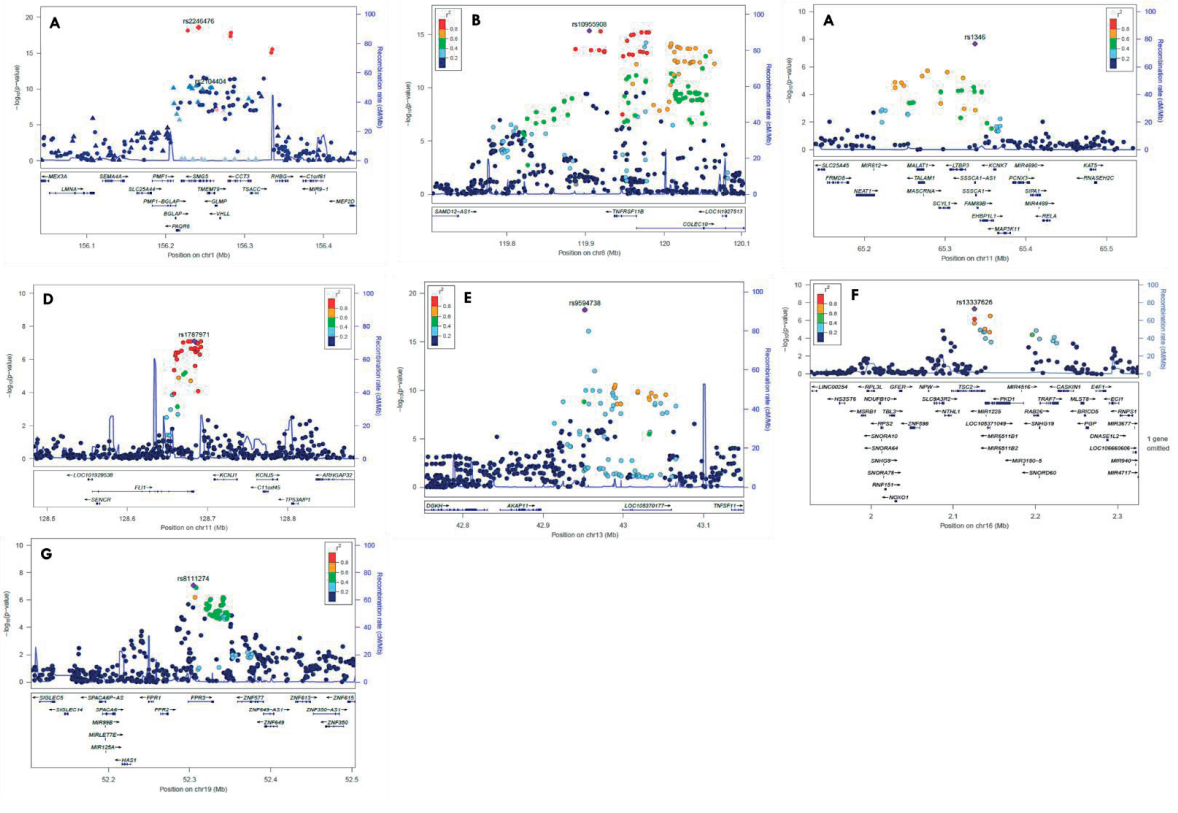
SUPPLEMENTARY MATERIAL



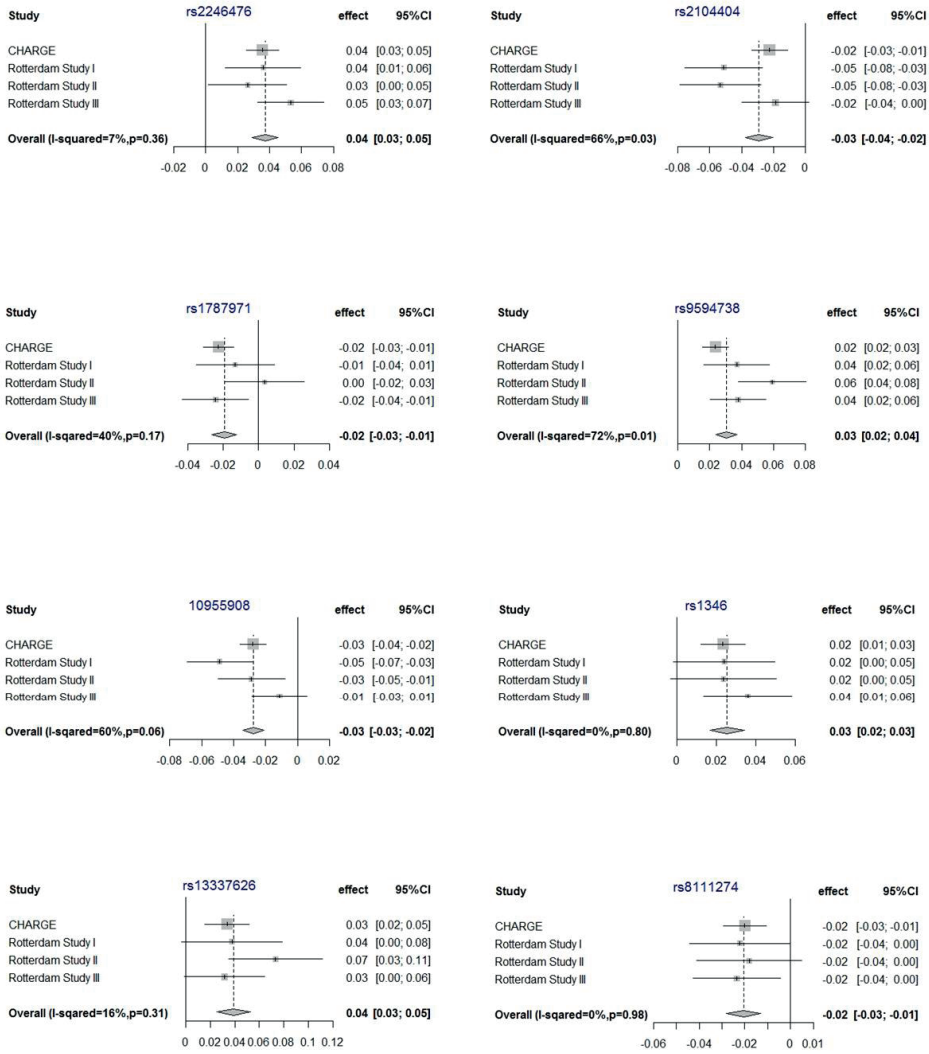
Supplementary Figure 1 | Manhattan Plot of Association Statistics (−log10(P)) for serum total osteocalcin. Each dot represents a SNP and the x axis indicates its chromosomal position (built 37 NCBI). Dashed red horizontal line marks the GWS threshold ($P \leq 5 \times 10^{-08}$).



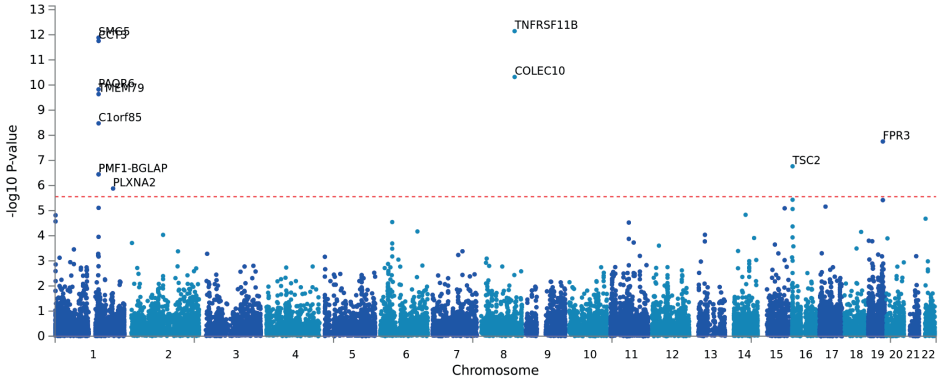
Supplementary Figure 2 | Quantile-quantile (QQ) plot of observed versus expected P values of the GWAS results. The dashed red line in the QQ plot indicates the distribution of SNPs under the null hypothesis.



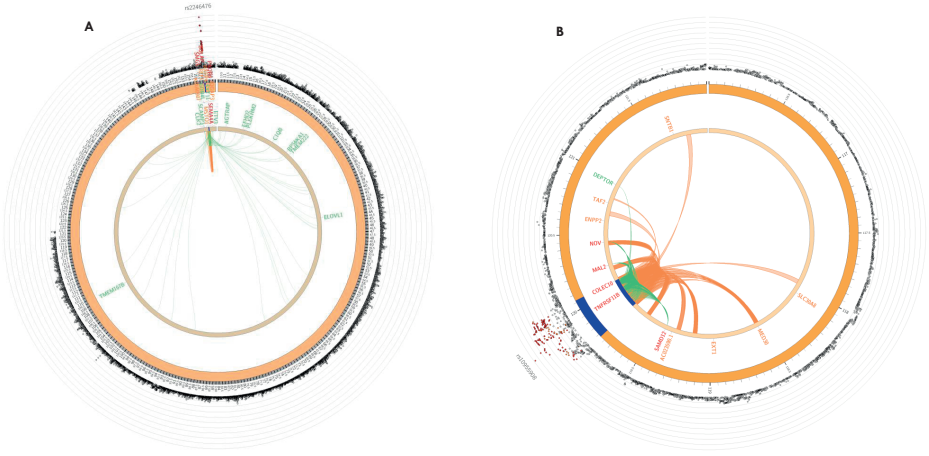
Supplementary figure 3 | Regional association plots displaying1q22 (A), 8q24.12 (B), 11q13.1(C), 11q24.3 (D), 13q14.11 (E), 16p13.3(F) and 19q13.41 (G) loci. In each plot, the −log10 of p values are on the left y-axis; the SNP genomic position (hg19) on the *x*-axis; the estimated recombination rate from 1000 genomes (March 2015 EUR) are on the right y-axis and plotted in blue. SNPs are coloured red to reflect linkage disequilibrium (LD) with the most significant SNP in purple (pairwise r2 from 1000 genomes March 2015 EUR).



Supplementary figure 4 | Forest plots displaying the effect estimates of the GWS SNPs for each individual study and the pool estimated. The effect estimate represents 1-unit change in the log transformed serum total osteocalcin per copy of the modelled allele.

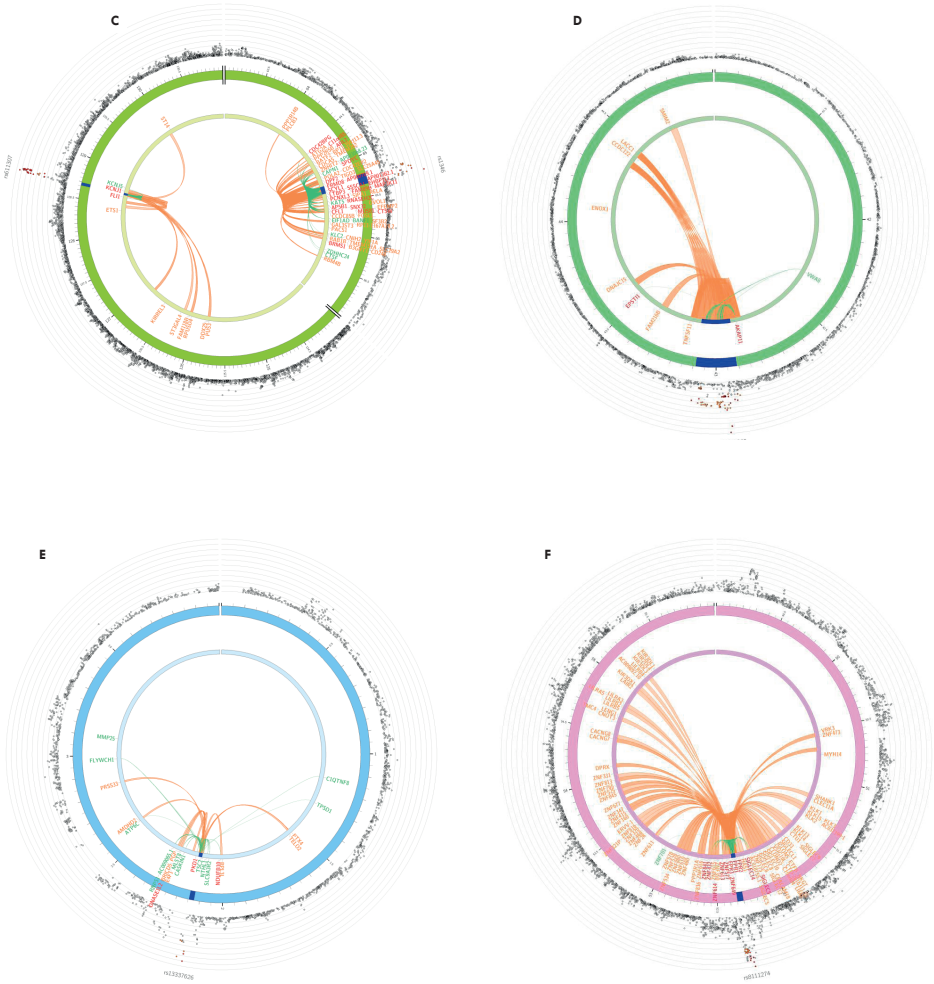


Supplementary figure 5 | Gene-Based Associaiton Analyses Using Summary Statistics from Discovery Meta-Analysis



Supplementary Figure 6 | The Circos Plot with Chromatin Inetraction (Orange lines) and eQTLs (Green Lines) on displaying1q22 (A), 8q24.12 (B), 11q13.1(C), 11q24.3 (D), 13q14.11 (E), 16p13.3(F) and 19q13.41 (G) loci.

The most outer layer represents the Manhattan plot. SNPs in genomic risk loci are colour-coded as a function of their maximum r2 to the lead SNPs in the locus, as follows: red (r2 > 0.8), orange (r2 > 0.6), green (r2 > 0.4), blue (r2 > 0.2) and grey (r2 ≤ 0.2). The rs ID of the top SNPs in the risk locus is displayed in the most outer layer. The second layer contains the genomic risk locus which is highlighted in blue. Here genes are mapped by chromatin interactions (**orange**) or eQTLs (**green**). When the gene is mapped by both, it is colored in red.



Supplementary Figure 6 | The Circos Plot with Chromatin Inetraction (Orange lines) and eQTLs (Green Lines) on displaying1q22 (A), 8q24.12 (B), 11q13.1(C), 11q24.3 (D), 13q14.11 (E), 16p13.3(F) and 19q13.41 (G) loci.

The most outer layer represents the Manhattan plot. SNPs in genomic risk loci are colour-coded as a function of their maximum r2 to the lead SNPs in the locus, as follows: red (r2 > 0.8), orange (r2 > 0.6), green (r2 > 0.4), blue (r2 > 0.2) and grey (r2 ≤ 0.2). The rs ID of the top SNPs in the risk locus is displayed in the most outer layer. The second layer contains the genomic risk locus which is highlighted in blue. Here genes are mapped by chromatin interactions (**orange**) or eQTLs (**green**). When the gene is mapped by both, it is colored in red. *(continued)*

Supplementary Table S2 | Genotype, Imputation and Sample QC information for Participating Cohorts

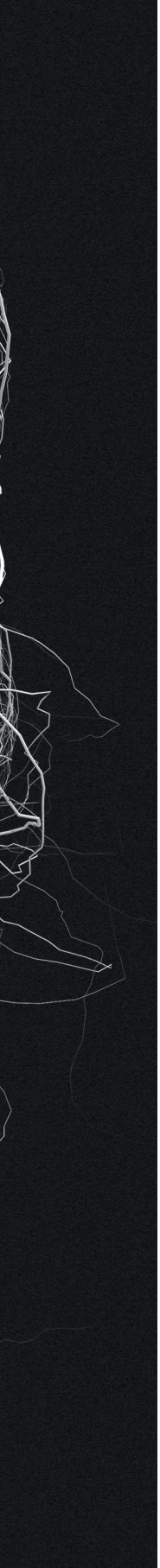
	Genotyping		SNP Inclusion criteria			
Study	**Platform(s) / Chip(s)**	**Calling Algorithm**	**MAF**	**Call Rate**	**P-test HWE**	**# SNPs**
			Discovery Cohorts			
AGEs	Illumina Hu370CNV	BeadStudio	≥1%	≥97%	$>10^{-6}$	308340
FHS	Affymatrix 550K	BRLMM	≥1%	≥97%	$>10^{-6}$	378163
GOOD	Illumina Infinium HumanHap 610K	BeadStudio	≥1%	≥98%	$>10^{-6}$	521160
HealthABC	Illumina Infinium 1M	BeadStudio	≥1%	≥97%	$>10^{-6}$	African American = 1007669. European Ancestry 914075
Mr.OS Sweden	Illumina HumanOmni1_Quad_v1-0 B	GenomeStudio	≥1%	≥98%	$>10^{-4}$ for autosomes. $>10^{-3}$ for X chromosome	739477
Mr.OS US	Illumina HumanOmni1_Quad_v1-0 B	GenomeStudio	≥ 1%	≥ 97%	≥ 10-4	725550
PIVUS	Human Omni Express	GenCall	>0	≥95%	$>10^{-6}$	645318
SHIP	Affymetrix 6.0	Birdseed2	NA	NA	NA	869224
SHIP-Trend	Illimuna Omni 2.5	GenCall	>0	>0.9	>0.0001	1782967
TwinsUK	HumanHap300 HumanHap610Q. 1M-Duo and 1.2MDuo 1M).	Illluminus	MAF>1%	SNP call rate >97% (SNPs with MAF≥5%) or > 99% (for 1% ≤MAF < 5%)	>10−6	317k=303.940; 610k=554.234; 1M= 878.319

	SNP Imputation			Samples	Association	
Method	**MAF**	**Quality metric**	**Total # SNPs**	**Call rate* selection**	**Sample QC / Other exclusions**	**Software**
				Discovery Cohorts		
MACH 1.0.16	>0%	r^2-hat≥0.30	2533153	≥97%	1) Mismatch with previous genotypes from other experiments. 2) missing height and weight	PorbABEL 0.0.5c
MACH 1.0.16	≥1%	$(O/E)\sigma^2$ ratio ≥0.30	2471285	≥97%	1) Autosomal heterozygosity (<0.26 or > 0.30); (2) Ethnic outliers (Eigenstrata)	Kinship. R-package
MACH 1.0.16	none	none	2543887	≥97.5%	1) heterozygosity > 33%; 2) ethnic outliers; 3) related individuals and duplicates.	MACH2qtl on GRIMP
MACH 1.0.16	none	none	African American = 1922501. European Ancestry 2543887	≥97%	No IBD > 0.125. no ethnic outliers from Eigenstrat. no gender discrepancies from self-report versus genotype data	MACH@ QTL
minimac	none	none		≥97%	exclusion based on IBD clustering. checked for duplicates. first and second-degree relatives	PLINK
minimac	none	MACH r2	3020488	≥ 97%	1) related participants and duplicates 2) High BAF in >5 chromosomes	R
IMPUTE 2.0	none	info>0	2692093	>95%	Heterozygosity (+-3SD. Gender discordance)	PLINK
IMPUTE	NA	excl. duplicate IDs	2748910	>92%	duplicate samples (by estimated IBD). individuals with reported/genotyped gender mismatch	Quicktest v0.95
IMPUTE	NA	excl. duplicate IDs	3437411	>94%	duplicate samples (by estimated IBD). individuals with reported/genotyped gender mismatch	Quicktest v0.95
IMPUTEv2	≥1%	info≥0.30	3044097	≥98%	Exclusion criteria were:(ii) heterozygosity across all SNPs ≥2 s.d. from the sample mean (iii) evidence of non-European ancestry as assessed by PCA comparison with HapMap3 populations; (iv) observed pairwise IBD probabilities suggestive of sample identity errors; (v). We corrected misclassified monozygotic and dizygotic twins based on IBD probabilities	GenABEL

Supplementary Table S3 | Comparing the IVW and ZW meta-analysis for the lead GWS SNPs from each locus

Locus	SNP	EA	NEA	EAF	Effect	se	P	Z-score	P
1q22	rs2246476	T	C	0.780	0.037	0.004	$2.64x10^{-19}$	8.97	$2.60x10^{-19}$
1q22	rs2104404	A	C	0.201	-0.029	0.004	$6.94x10^{-11}$	-6.47	$1.01x10^{-10}$
8q24.12	rs10955908	A	C	0.467	-0.028	0.003	$4.53x10^{-16}$	-8.21	$2.18x10^{-16}$
11q13.1	rs1346	A	T	0.812	0.025	0.005	$2.16x10^{-8}$	5.61	$1.95x10^{-8}$
11q24.3	rs1787971	C	G	0.670	-0.020	0.004	$7.73x10^{-8}$	-5.34	$9.18x10^{-8}$
13q14.11	rs9594738	T	C	0.498	0.030	0.003	$5.04x10^{-19}$	8.93	$4.36x10^{-19}$
16p13.3	rs13337626	T	C	0.916	0.039	0.007	$4.82x10^{-8}$	5.38	$7.40x10^{-8}$
19q13.41	rs8111274	T	G	0.291	-0.021	0.004	$8.25x10^{-8}$	-5.37	$7.70x10^{-8}$





2.2.3

Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects

Carolina Medina-Gomez*, John P Kemp*, Katerina Trajanoska, Jian'an Luan, Alessandra Chesi, Tarunveer S Ahluwalia, Dennis O Mook-Kanamori, Annelies Ham, Fernando P Hartwig, Daniel S Evans, Raimo Joro, Ivana Nedeljkovic, Hou-Feng Zheng, Kun Zhu, Mustafa Atalay, Ching-Ti Liu, Maria Nethander, Linda Broer, Gudmar Porleifsson, Benjamin H Mullin, Samuel K Handelman, Mike A Nalls, Leon E Jessen, Denise H M Heppe, J Brent Richards, Carol Wang, Bo Chawes, Katharina E Schraut, Najaf Amin, Nick Wareham, David Karasik, Nathalie Van der Velde, M Arfan Ikram, Babette S Zemel, Yanhua Zhou, Christian J Carlsson, Yongmei Liu, Fiona E McGuigan, Cindy G Boer, Klaus Bønnelykke, Stuart H Ralston, John A Robbins, John P Walsh, M Carola Zillikens, Claudia Langenberg, Ruifang Li-Gao, Frances M K Williams, Tamara B Harris, Kristina Akesson, Rebecca D Jackson, Gunnar Sigurdsson, Martin den Heijer, Bram C J van der Eerden , Jeroen van de Peppel, Timothy D Spector, Craig Pennell, Bernardo L Horta, Janine F Felix, Jing Hua Zhao, Scott G Wilson, Renée de Mutsert, Hans Bisgaard, Unnur Styrkársdóttir, Vincent W Jaddoe, Eric Orwoll, Timo A Lakka, Robert Scott, Struan F A Grant, Mattias Lorentzon, Cornelia M van Duijn, James F Wilson, Kari Stefansson, Bruce M Psaty, Douglas P Kiel, Claes Ohlsson, Evangelia Ntzani, Andre J van Wijnen, Vincenzo Forgetta, Mohsen Ghanbari, John G Logan, Graham R Williams, J H Duncan Bassett, Peter I Croucher, Evangelos Evangelou, Andre G Uitterlinden, Cheryl L Ackert-Bicknell, Jonathan H Tobias, David M Evans**, Fernando Rivadeneira**

*Denotes equal contribution

**Denotes equal supervision

Am J Hum Genet. *2018 Jan 4;102(1):88-102.*

ABSTRACT

Bone mineral density (BMD) assessed by DXA is used to evaluate bone health. In children, total body (TB) measurements are commonly used; in older individuals, BMD at the lumbar spine (LS) and femoral neck (FN) is used to diagnose osteoporosis. To date, genetic variants in more than 60 loci have been identified as associated with BMD. To investigate the genetic determinants of TB-BMD variation along the life course and test for age-specific effects, we performed a meta-analysis of 30 genome-wide association studies (GWASs) of TB-BMD including 66,628 individuals overall and divided across five age strata, each spanning 15 years. We identified variants associated with TB-BMD at 80 loci, of which 36 have not been previously identified; overall, they explain approximately 10% of the TB-BMD variance when combining all age groups and influence the risk of fracture. Pathway and enrichment analysis of the association signals showed clustering within gene sets implicated in the regulation of cell growth and SMAD proteins, overexpressed in the musculoskeletal system, and enriched in enhancer and promoter regions. These findings reveal TB-BMD as a relevant trait for genetic studies of osteoporosis, enabling the identification of variants and pathways influencing different bone compartments. Only variants in ESR1 and close proximity to RANKL showed a clear effect dependency on age. This most likely indicates that the majority of genetic variants identified influence BMD early in life and that their effect can be captured throughout the life course.

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased risk of fracture.[1] It is diagnosed through the measurement of bone mineral density (BMD) utilizing dual-energy X-ray absorptiometry (DXA), which is the single best predictor of fracture.[1]

Bone is a dynamic tissue constantly undergoing resorption and formation. Bone mass increases steadily during childhood and markedly during adolescent growth.[2] Peak bone mass is attained at approximately the third decade of life. Thereafter, until about 50 years of age, BMD remains fairly stable, by virtue of the coupling between bone formation and resorption (e.g., bone remodeling). Subsequently, bone resorption exceeds the rate of bone formation, resulting in a decrease in BMD, particularly in women after the onset of menopause.[3] The International Society for Clinical Densitometry (ISCD) recommends performing DXA measurements at the lumbar spine, femoral neck, and total hip to diagnose osteoporosis in postmenopausal women and men who are 50 years or older (see Web Resources). Consequently, studies of BMD determinants are frequently based on measurements at these skeletal sites. By contrast, for the assessment of bone health in children and adolescents, total body (excluding head) and lumbar spine are the preferred sites to minimize measurement artifacts resulting from changing areas in growing bones (**see Web Resources**). Nevertheless, in elderly individuals' degenerative changes in the spine can give elevated BMD readings.[4] Moreover, total body DXA scans have been obtained in many adult research cohorts, primarily to assess body composition. Therefore, the total body BMD (TB-BMD) measurement is the most appropriate method for an unbiased assessment of BMD variation in the same skeletal site from childhood to old age. To date, nearly 80 independent genetic variants have been shown to be robustly associated with variability in bone parameters.[5–17] Most of these markers have been identified in studies comprising tens of thousands of adult and elderly individuals with DXA-derived BMD measurements, although a few of them have been associated with BMD specifically in studies of pediatric cohorts.[7] Furthermore, several of the associated variants display significant site-specific effects, possibly reflecting differences in bone composition across skeletal sites (e.g., cortical bone versus trabecular bone) or differential response to mechanical loading.[7] Moreover, genetic studies on measures from peripheral quantitative computed tomography (pQCT) and bone quantitative ultrasound, which provide additional information regarding bone size, geometry, and (micro) architecture, identified genetic variants that may have specific effects on bone properties that are poorly captured by conventional DXA measurements.[8,9]

Given the complex physiological processes underlying age-related changes in BMD across the life course, it is possible that genetic studies in more refined age groups

will reveal variants in unreported loci as well as age-specific genetic effects. Thus, the purpose of this study was to identify gene variants associated with TB-BMD across the lifespan and to investigate possible differences of genetic effects across age periods.

SUBJECTS AND METHODS

TB-BMD GWAS meta-analyses

Subjects, BMD Measurement, and Imputation

This study comprised 30 epidemiological studies comprising 66,628 individuals from populations across America, Europe, and Australia, with a variety of designs (**Supplemental Data; Suplementary Table 1**) and participant characteristics (**Suplementary Table 2**). In summary, most participants came from population-based cohorts of European ancestry (86%), two cohorts comprised African American individuals (2%), and four other studies held a fraction of individuals from admixed background (14%). All research aims and the specific measurements have been approved by the correspondent Medical Ethical Committee of each participating study. Written informed consent was provided by all subjects or by their parents in the case of children.

Total body BMD (g/cm^2) was measured by DXA following standard manufacturer protocols. As recommended by the International Society for Clinical Densitometry, total body less head (TBLH) was the measurement used in pediatric cohorts (see Web Resources) (e.g., 0–15 years). Detailed information on the assessments performed by each study can be found in **Suplementary Table 1**.

All individuals included in this study had genome-wide array data. Quality control of genotypes is summarized in **Suplementary Table 1**. To enable meta-analysis, each study performed genotype imputation using the cosmopolitan (all ethnicities combined) 1000 Genomes phase 1 v.3 (March 2012) reference panel, yielding 30,000,000 SNPs for analysis. Three studies used the combined 1000 Genomes and the UK10K reference panels as presented in **Suplementary Table 1.**

Association Analysis, Quality Control, and Assessment

TB(LH)-BMD was corrected for age, weight, height, and genomic principal components (derived from GWAS data), as well as any additional study-specific covariates (e.g., recruiting center), in a linear regression model. For studies with non-related individuals, residuals were computed separately by sex, whereas for family-based studies sex was included as a covariate in the model. Finally, residuals were inverse normal transformed. The analyses were performed in each study for the overall population as well as in subgroups of individuals by age strata, defined by bins of 15 years (i.e., 0-15

years, 15-30 years, 30-45 years, 45-60 years, and 60 or more years). SNP association was tested for autosomal variants, in which the additive effect of each SNP on the normalized BMD residuals was estimated via linear regression.

A centralized quality-control procedure implemented in EasyQC[18] was applied to all study-specific files of association results to identify cohort-specific issues. We excluded variants if they had missing information (e.g., missing association p value, beta estimate, alleles, allele frequency), nonsensical values (e.g., absolute beta estimates or standard errors > 10, association p values > 1 or < 0, imputation quality<0, infinite beta estimates or standard errors), minor allele frequency (MAF) less than 0.5%, or imputation quality scores < 0.4 (Impute2) or < 0.3 (Minimac). Moreover, variants were flagged if they had large allele frequency deviations from reference populations (>0.6 for admixed studies and >0.3 for ancestry-homogeneous studies).

In the first instance, no exclusion criteria based on ancestry were applied for the meta-analysis (N = 66,628). In addition, meta-analyses were carried out across age strata (minimum sample size per bin N =200 for each study) comprising 0-15 years (N=11,807), 15-30 years (N=4,180), 30-45 years (N=10,062), 45-60 years (N=18,805), and 60 or more years (N= 22,504). Further, summary data from cohorts of European ancestry only were meta-analyzed and used in subsequent analyses. We discarded variants present in fewer than three studies. Approximately 23,700,000 markers (including SNPs and INDELS) were assessed for association. We applied the conventional genome-wide significance level (GWS, $p<5x10^{-8}$) for SNP discovery.

We selected SNPs that were suggestively (12,567 SNPs, $p < 5x10^{-6}$) associated with BMD in the overall meta-analysis, present in at least two studies per age bin, and with MAF differences across these meta-analyses lower than 0.5. We clumped this dataset with an $r^2 \geq 0.8$, using as reference the most strongly associated SNPs with BMD and pruning remaining SNPs within 0.7 Mb of each other. Age-dependent effects were assessed using a meta-regression approach for 1,464 SNPs obtained after this selection procedure. We ran a linear regression of the SNP effect estimates onto an intercept and the median age of each subgroup (e.g., each study stratified in age bins). As pro-posed previously,[19] standard errors of the effect estimates of each subgroup were multiplied by the square root of the genomic inflation factor when it was greater than 1. We performed the meta-regression using the Metafor package,[20] and any statistical evidence of linear association was corrected for multiple testing (Bonferroni correction; $0.05/ 1,464 = 3.4x10^{-5}$). The difference between beta-estimates in children versus elderly meta-analyses (P_{diff}) was tested using Easy-strata.[21]

Conditional analyses were undertaken based on the meta-analysis of the studies of European ancestry only (N =56,284). Only variants in the loci that reached GWS in this meta-analysis were assessed. The Rotterdam Study I (n =6,291) was used as reference for precise calculation of the linkage disequilibrium (LD) between the analyzed

markers. We used an iterative strategy as implemented in GCTA[22] to determine (1) independence of association signals within loci discovered in our study, by means of stepwise model selection procedure per chromosome (-massoc-slct routine) and (2) the novelty of the association signals discovered by our meta-analysis with regard to variants reported in previous well-powered GWASs of different bone traits (**Supplementary Table 3**). To this end, we performed the association analysis conditional on 78 variants present in our data and associated with different bone traits (-massoc-cond routine). These 78 SNPs were selected from different GWAS publications,[5–9,11–13] assuring their independence to avoid collinearity issues.

Shared Genetic Architecture of TB-BMD Fracture and Other Traits

LD Score Regression Analyses

We used the LD score regression package to estimate the heritability of TB-BMD and to rule out that our results were a product of bias (e.g., residual population stratification or cryptic relatedness). LD score regression uses GWAS summary statistics and assesses the SNP heritability based on the expected relationship between LD of neighboring SNPs and strength of association under a polygenic model.[23] As this methodology relies on the LD structure throughout the genome, we restricted this analysis to summary statistics from the meta-analysis of cohorts comprising only individuals from European ancestry. We used the publicly available, pre-computed LD structure data files specific to European populations of the HAPMAP 3 reference panel. An extension of this method allows estimating the genetic correlation between two traits.[24] This can be performed in the LDhub pipeline, a web utility that gathers data from many different GWAS meta-analysis.[25] From the 199 traits, currently available in the website, we have restricted our analysis to those traits whose heritability z-scores were larger than 4 and were analyzed only in European ancestry individuals (following the recommendations in the LD score software website [Web Resources]). Additionally, we incorporated data from a recent GWAS meta-analysis of any type of fracture in individuals from European ancestry (N = 264,267; 37,778 case subjects) (K.T., unpublished data). In total, we assessed the genetic correlation between TB-BMD and 74 traits.

Mendelian Randomization Analysis

We undertook a two-sample Mendelian randomization approach[26] to estimate the causal effect of TB-BMD on any type of fracture in the Europeans samples. In short, we constructed a score based on the independent genetic variants from the TB-BMD meta-analysis (European set and excluding secondary signals); whenever the selected variant was not present in the fracture meta-analysis, the second variant with the

lowest p value in the locus ($p < 5x10^{-8}$) and $r^2 > 0.8$ was used as proxy. Thereafter, estimates derived from the TB-BMD summary statistics were pooled using methods similar to inverse-variance weighted fixed meta-analysis using the meta R-package (Web Resources).

Search for Biological and Functional Knowledge of the Identified Association Regions

For all those SNPs outside a 500 kb window from previously known bone-associated SNPs, we did a literature search in PubMed and Web of Science to evaluate whether nearby genes (within 500 kb) were known to play a role in bone metabolism. Also, we determined whether the annotated genes underlie any human Mendelian disorder with a skeletal manifestation, had knockout mouse models with a skeletal phenotype, or were annotated to pathways critical to bone metabolism. Genomic annotation for all SNPs was made based on UCSC hg19.

DEPICT Analyses

We used DEPICT,[27] a recently developed tool to prioritize genes at the associated regions, define possible pathways by enrichment testing, and identify tissue and cell types in which genes from loci associated with TB-BMD. The methodology first selects all lead SNPs below a certain threshold with respect to a target p value. We tested both the complete set of GWS SNPs and the subset of those mapping only to loci not previously reported. Enriched gene sets were group based on the degree of gene overlap into ''meta gene-sets'' as proposed earlier,[28] and their correlation visualized using Cytoscape 3.4 (Web Resources).

Functional Annotation to MicroRNA Binding Sites

We used the PolymiRTS,[28] miRdSNP,[29] and microSNiPer[30] databases to obtain a list of variants located in predicted microRNA binding sites on the 3′ UTRs of genes, as described in detail elsewhere.[31] In summary, index SNPs (most associated variants) of the GWS loci were submitted to SNAP (**Web Resources**) to retrieve their high LD proxy SNPs (with $r^2 > 0.8$, limit distance 500 kb, and CEU panel) in the 1000 Genomes project. The resulting list of SNPs was annotated to the list of microRNA binding site variants obtained from the above mentioned publicly available databases.

Functional Enrichment Analysis of Trait-Associated Variants

GWAS analysis of regulatory or functional information enrichment with LD correction (GARFIELD)[32] was used to characterize the putative functional contribution of TB-BMD-associated variants mapping to non-coding regions. GARFIELD employs a nonparametric analysis to calculate fold enrichment values for regulatory marks, at

given significance thresholds, and then tests them for significance via permutation testing while accounting for LD, MAF, and local gene density.[32] We used data regarding DNase-I hypersensitive sites, transcription factor binding sites, histone modifications, and chromatin states (ENCODE and Roadmap Epigenomics) from 424 cell types and tissues to capture and characterize possible cell-type-specific patterns of enrichment, as provided in the GARFIELD software (Web Resources). Fold enrichment statistics were tested at the four different significance thresholds (i.e., $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, and $1x10^{-5}$). Multiple-testing correction was performed on the effective number of annotations used, using the default p value threshold of $1x10^{-4}$.

Knockout Animal Models and Gene Expression in Bone Cells

Animal Models Survey

We surveyed databases from The International Mouse Phenotyping Consortium[33] together with The International Knockout Mouse Consortium[34] to identify knockout models of candidate genes resulting in skeletal phenotypes. Furthermore, we mined data from The Origins of Bone and Cartilage Disease (OBCD) project,[35] specialized in murine skeletal phenotypes including digital X-ray microradiography on femurs and tail vertebrae, micro-CT analysis, femur three-point bend test load-displacement curves, and tail vertebrae compression testing from knockout mice and wild-type controls at 16 weeks of age. Experiments were undertaken by the Wellcome Trust Sanger Institute Mouse Genetics Project as part of the IKMC and licensed by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and the recommendations of the Weatherall report.

Gene Expression in Bone Cells

Gene expression profiles of candidate genes were examined in primary mouse osteoblasts undergoing differentiation and bone marrow-derived osteoclasts. To study murine osteoblasts, pre-osteoblast-like cells were obtained from neonatal calvaria collected from C57BL/6J. Next Generation RNA sequencing using an Illumina HiSeq 2000 was used to evaluate the transcriptome every 2 days from day 2 to 18 days after osteoblast differentiation.[6] Expression of genes in murine osteoclasts was determined using publicly available data obtained using Next-Gen RNA-sequencing applied to bone marrow-derived osteoclasts obtained from 6- to 8-week-old C57BL/6 mice.[36] All procedures and use of mice for the neonatal osteoblast expression studies were approved by the Jackson Laboratory Animal Care and Use Committee (ACUC), in accordance with NIH guidelines for the care and use of laboratory animals. Gene expression profiles of candidate genes were examined in human bone marrow-derived mesenchymal stem cells differentiated into osteoblast. Total RNA (n = 3) was isolated

at day 0 (MSCs) and day 4 of osteoblast differentia-tion.[37] Also, RNA was isolated during osteoclast differentiation. Peripheral blood mononuclear cells derived from buffy coats (Sanquin) were seeded in 96-well plates ($5x10^{-5}$ cells per well) as previously described.[38] Total RNA (n = 3) was isolated using Trizol at day 0 (PBMCs) and at day 7 of osteoclast differentiation. Illumina Hu-manHT-12 v3 BeadChip human whole-genome expression arrays were used for expression profiling. The quality of isolated RNA was assessed on a 2100 Bioanalyzer (Agilent Tech-nologies). Data were analyzed as described in detail previously.[37] Genes were designated as being expressed when at least one probe coding for the gene was significantly present in at least two of the three biological replicates.

RESULTS

TB-BMD GWAS Meta-analyses

Analyses Including All Age Strata

Our meta-analysis of TB-BMD GWAS summary statistics (N = 66,628) identified variants in 76 independent loci associated with TB-BMD at a genome-wide significant (GWS, $P \leq 5x10^{-8}$) level (**Figure 1, Suplementary Table 4**). Overall, there was no evidence of a strong inflation (genomic inflation factor [l] of 1.08, **Suplementary Figure 1**). Yet, inflation was observed in the range of common variants (0.2 > MAF < 0.5, l =1.19)

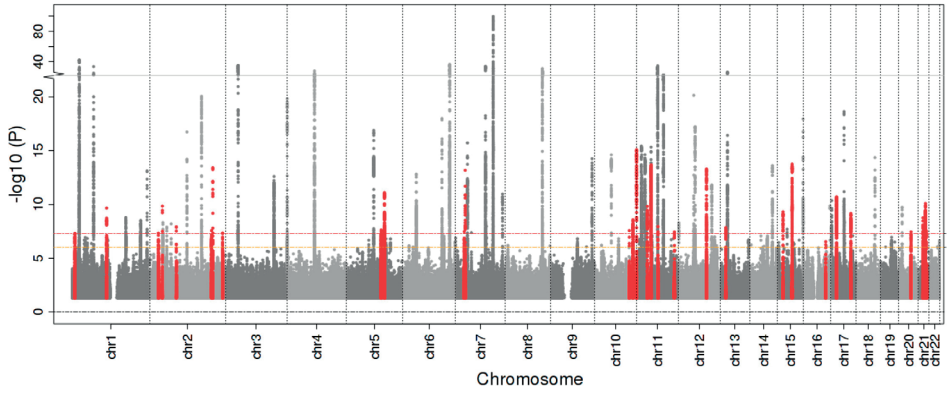


Figure 1 | Manhattan Plot of Association Statistics (log10(p Values)) for TB-BMD Overall Meta-analysis. Each dot represents a SNP and the x axis indicates its chromosomal position (built 37 NCBI). Red dots represent SNPs at GWS loci that are not within 5500 kb of leading SNPs in previous GWASs with different bone traits. Dashed horizontal red and yellow lines mark the GWS threshold ($p < 5x10^{-8}$) and suggestive threshold ($p < 1x10^{-6}$), respectively. Novel loci in the only-CEU analysis are not shown.

due to polygenicity (LD score regression intercept =1.007). In our results, one of the signals mapping to LDLRAD3 was driven entirely by individuals of African background (MAF = 0.043 in YRI panel) since the two associated variants are monomorphic in all other populations. The low allele frequency of this variant in our study (MAF = 0.025) and our limited statistical power (N = 6,748) in non-European samples warrants independent replication efforts to exclude the possibility of a false-positive association. In addition, a meta-analysis comprising 56,284 individuals of European ancestry (84% of the study population) identified variants in two additional GWS loci (**Suplementary Figures 1** and **2, Suplementary Table 5**). Association signals mapping to these loci were close to the GWS threshold in the overall meta-analysis ($p=1x10^{-7}$) and showed no evidence of heterogeneity ($p_{het} > 0.1$). One of them, in 12q24.21 (*MED13L*), has not been previously associated with bone parameters **(Table 1, Suplementary Figure 3**), while the other in 21q22.13 (*CLDN14*) is not fully independent from the previously reported hip BMD association signal[12] (**Suplementary Table 5**).

Of the 78 identified loci, variants in 35 (45%) were not located within 500 kb of known association signals nor in regions of extended LD with them (**Table 1, Suplementary Figure 4**). Index SNPs at these 35 loci were, in general, common non-coding variants. Twenty-two of these are located in close proximity to genes likely to influence bone metabolism as shown by previous functional studies (**Table 1, Suplementary Figure 3**), including CSF1 (MIM: 120420), important for osteoclast differentiation,[39] and SMAD3 (MIM: 603109), a critical component of the TGF-b signaling pathway.[56] Across these 35 signals, 31 of the index SNPs were nominally associated ($p < 0.05$) with either lumbar spine or femoral neck BMD in the same direction as in the previously published GEFOS GWAS meta-analysis[6] (**Table 1**). This comparison was not possible for the rs113964474 variant, because it was not available in the GEFOS study. Moreover, we found directionally concordant effect estimates ($p < 0.05$) for 73 of the 78 index SNPs of known bone association signals (**Suplementary Table 3**). The markers that failed to replicate in our study were either previously associated with lumbar spine BMD but not femoral neck BMD (rs3905706 *[MPP7*, 10p12.1] and rs1878526 *[INSIG2*, 2q14.2]), associated specifically with the hip trochanter and intertrochanteric sub-regions (rs1949542 [*RP11-384F7.1*, 3q13.32]), or associated with BMD only in women (rs7017914 [XKR9, 8q13.3]) or only in children (rs754388 [*RIN3*, 14q32.12]).

Table 1 | Index SNPs of Loci Not Previously Associated with BMD

rsnumber	Locus	AA1	A2	EAF	Effect	P	N	annotation	closest gene	Notes	LS-beta	LS-P	FN-beta	FN-P
rs2252865	1p36.23	T	C	0.32	-0.033	$4.72x10^{-8}$	66075	intronic	*RERE*	Novel biology	-0.019	0.043	-0.025	0.002
rs7548588	1p13.3	T	C	0.61	-0.037	$9.29x10^{-9}$	66240	intergenic	*CSF1*	Osteoclast differentiation (19)	-0.030	0.001	-0.022	0.005
rs185048405	1q41	T	C	0.54	0.042	$3.07x10^{-9}$	66540	intronic	*SLC30A10*	Manganese transport. (24)	-0.035	0.076	-0.003	0.878
rs780096	2p23.3	C	G	0.44	-0.031	$4.58x10^{-8}$	66578	introinc	*GCKR*	Calcium serum regulation (25)	-0.014	0.129	-0.017	0.029
rs10490046	2p22.1	A	C	0.76	0.043	$1.43x10^{-10}$	65961	intron	*SLC8A1*	Bone mineralization (26)	0.015	0.162	0.021	0.025
rs10048745	2p13.3	A	G	0.25	-0.039	$6.44x10^{-9}$	66565	5'-UTR	*ARHGAP25*	Novel biology	-0.050	$1.03x10^{-6}$	-0.036	$5.21 x10^{-5}$
rs11904127	2p11.2	A	G	0.55	-0.032	$2.65x10^{-8}$	66561	intronic	*TCF7L1*	Factors in Wnt signaling (27)	-0.021	0.023	-0.015	0.054
rs1595824	2q33.1	T	C	0.47	0.034	$2.65x10^{-8}$	60171	intronic	*PLCL1*	Negative regulation of bone formation (28)	0.022	0.201	0.052	$2.20x10^{-4}$
rs2350085	2q33.2	T	C	0.87	-0.064	$3.80x10^{-14}$	66412	intergenic	*FZD7*	Factors in Wnt signaling (29)	-0.042	0.002	-0.044	$1.96x10^{-4}$
rs838721	2q37.1	A	G	0.44	-0.031	$4.48x10^{-9}$	65516	intronic	*DGKD*	Clacium serum regulation (25)	-0.016	0.070	-0.014	0.068
rs818427	5q22.2	T	C	0.31	0.034	$2.37x10^{-8}$	66592	intronic	*APC*	Bone metabolism (30)	0.004	0.645	0.008	0.327
rs11745493	5q23.2	A	G	0.75	0.044	$7.75x10^{-12}$	66597	promoter	*CSNK1G3*	Novel Biology	0.010	0.326	0.025	0.005
rs757138	7p15.1	T	G	0.69	-0.035	$3.33x10^{-8}$	66043	intronic	*JAZF1*	Novel Biology	-0.016	0.126	-0.025	0.004
rs28362721	7p14.3	T	C	0.18	-0.059	$6.71x10^{-14}$	66274	intronic	*AQP1*	Bone metabolism (31)	-0.037	0.002	-0.049	$1.39x10^{-6}$
rs1548607	7p12.1	A	G	0.69	0.036	$4.18x10^{-8}$	66564	intergenic	*GRB10*	Novel biology	0.034	$5.59x10^{-4}$	0.005	0.517
rs34670419	7q22.1	T	G	0.04	-0.088	$1.09x10^{-8}$	66336	3'-UTR	*ZKSCAN5*	DHEAS and aging mechanisms (53)	-0.127	$9.2 x10^{-8}$	-0.080	$8.19x10^{-5}$
rs73349318	10q25.2	A	T	0.87	-0.047	$2.68x10^{-8}$	66341	intronic	*DUSP5*	Osteoclast differentiation (32)	-0.042	0.001	-0.051	$8.76x10^{-6}$
rs10788264	10q26.13	A	G	0.48	-0.034	$2.61x10^{-9}$	66565	intergenic	*TACC2*	Novel Biology	-0.030	$9.64x10^{-4}$	-0.029	$1.29x10^{-4}$
rs55781332	11p15.5	A	G	0.78	-0.055	$8.07x10^{-16}$	66198	intronic	*PSMD13*	Novel Biology	-0.046	$1.7 x10^{-5}$	-0.026	0.005
rs2553773	11p13	C	G	0.41	-0.037	1.49x10-^{10}	66619	intergenic	*CD44*	Osteoclast activity (33)	-0.015	0.101	-0.015	0.054
rs113964474*	11p.13*	A	G	0.03	0.485	$1.41x10^{-8}$	6748	intron	*LDLRAD3*	Novel Biology	.	.	.	.
rs4980659	11q13.3	C	G	0.52	0.033	$1.16x10^{-8}$	66537	intergenic	*CCND1*	Target of Wnt signalling (42)	0.039	$1.58x10^{-5}$	0.023	0.003
rs725670	11q24.1	A	G	0.38	-0.032	$3.61x10^{-8}$	66565	intergenic	*BLID*	Novel Biology	-0.020	0.028	-0.011	0.172

Table 1 | Index SNPs of Loci Not Previously Associated with BMD (*continued*)

rsnumber	Locus	AA1	A2	EAF	Effect	P	N	annotation	closest gene	Notes	LS-beta	LS-P	FN-beta	FN-P
rs10777212	12q21.33	T	G	0.35	0.045	$5.05x10^{-14}$	66619	intergenic	*ATP2B1*	Calcium absorption (35)	0.028	0.003	0.021	0.010
rs73200209**	12q24.21	A	T	0.80	0.045	$2.51x10^{-8}$	51240	intronic	*MED13L*	Novel biology	0.030	0.167	0.036	0.044
rs556429	13q13.3	A	C	0.23	0.039	$1.46x10^{-8}$	66504	intronic	*SMAD9*	Osteoblast differentiation (40)	0.023	0.027	0.013	0.135
rs12442242	15q14	A	G	0.85	-0.051	$4.94x10^{-10}$	66403	intergenic	*TMCO5A*	Novel Biology	-0.046	$3.03x10^{-4}$	-0.047	$2.26x10^{-5}$
rs2414098	15q21.2	T	C	0.39	-0.033	$1.99x10^{-8}$	66562	intronic	*CYP19A1*	Estrogen byosynthesis (50)	-0.034	0.007	-0.038	0.001
rs1545161	15q22.33	A	G	0.56	0.041	$1.06x10^{-12}$	66004	intron	*SMAD3*	Osteoblast differentiation (20.36)	0.034	$1.27x10^{-4}$	0.035	$5.78x10^{-5}$
rs8070128	17p11.2	T	C	0.58	-0.039	$1.98x10^{-11}$	66625	intronic	*TOM1L2*	Novel biology	-0.033	$4.80x10^{-4}$	-0.015	0.052
rs9972944	17q24.1	A	G	0.41	0.036	$6.87x10^{-10}$	66595	intron	*CEP112*	Novel Biology	0.028	0.003	0.004	0.576
rs6510186***	19q12	T	C	0.26	0.068	$3.11x10^{-8}$	18782	intergenic	*TSHZ3*	Novel Biology	0.004	0.713	0.006	0.492
rs6029130	20q12	T	C	0.30	0.035	$3.50x10^{-8}$	66497	intergenic	*MAFB*	Osteoclast differentiation (37)	0.027	0.007	0.015	0.083
rs1452102	21q21.3	T	G	0.59	-0.035	$1.74x10^{-9}$	66489	intergenic	*ADAMTS5*	Endochondral Ossification (41)	-0.029	0.001	-0.015	0.056
rs9976876	21q22.12	T	G	0.45	-0.038	$8.01x10^{-11}$	66514	intron	*RUNX1*	osteoclast differentiation (38)	-0.019	0.031	-0.016	0.041
rs11910328	21q22.2	A	G	0.84	-0.043	$2.99x10^{-8}$	66298	intergenic	*ETS2*	Osteoblast maturation (39)	-0.028	0.020	-0.028	0.007

Age-Dependent Effects

Meta-analyses across age strata resulted in the identification of variants mapping to two additional loci that were not detected in the overall meta-analysis (**Figure S5; Supplementary Table 6**). In children (age group 0-15 years), the previously known 14q32.12 locus,[7] harboring *RIN3* (rs72699866, p= $1x10^{-8}$), and in the middle-aged (age group 45–60 years), a signal in the 19q12 locus mapping in the vicinity of *TSHZ3* (rs6510186, p = $3.1x10^{-8}$) were identified. The rs72699866 variant leading the R*IN3* signal in the youngest age stratum showed no evidence of association (p = 0.16) and high heterogeneity (p_{het} = $6.6x10^{-5}$) in the overall meta-analysis. In fact, the effect of rs72699866 decreased significantly with age (p_{trend} = $1.69x10^{-9}$) (**Supplementary Figure 6**) and showed a significant difference between the two extreme groups, i.e., children versus elderly (b0-15 = 0.099 [0.066, 0.134]; b>60 = 0.035 [0.060, 0.010]; p_{diff} = $4.32\ 3x10^{-10}$). In contrast, the rs6510186 variant (19q12) showed nominal evidence of association and heterogeneity in the overall meta-analysis (p=0.02; p_{het}=0.03). Nevertheless, no clear pattern of age dependency was identified (p=0.2) for this SNP (**Supplementary Figure 6**). We also applied meta-regression analysis and found that variants mapping to 42 different loci showed nominally significant age-dependent effect (p < 0.05) (**Supplementary Table 7; Supplementary Figure 7**). In summary, 27 (64%) of the loci showed stronger effects in the older age groups. Of these variants in the 6q25.1 (*ESR1*) and 13q14.11 (*RANK*L) loci remained significant after multiple-testing correction (p < $3.4x10^{-5}$) (**Figure 2**), while variants in 6p21.1 (*RUNX2*, rs148460475), 15q21.2 (*CYP19A1*, rs2414098), 17q21.31 (*MEOX1*, rs74835612), and 11p15.1 (*SOX6*, rs11822790) were only suggestive at p < $1x10^{-3}$.

Conditional Association Analyses

The stepwise conditional approach included studies comprising only individuals of European ancestry, as the method used relies on appropriate representability of the LD reference. Of the 76 GWS loci identified in the overall analysis, variants in 57 (19 previously unreported) loci were also GWS in the European-only analysis (**Supplementary Figure 2**), likely a consequence of the lower power in this subgroup. We identified 81 SNPs independently associated with TB-BMD mapping to 58 different loci (one European-specific), 18 of which depicted multiple distinct signals attaining GWS (**Supplementary Table 8**). These independent variants together explained 10.2% of TB-BMD variance. This proportion is slightly higher than the 7.4% TB-BMD variance explained by the 78 known variants associated with bone traits. Moreover, we identified independent signals in 13 of the 78 known bone loci after conditional analyses **(Supplementary Figure 2; Supplementary Table 8**).

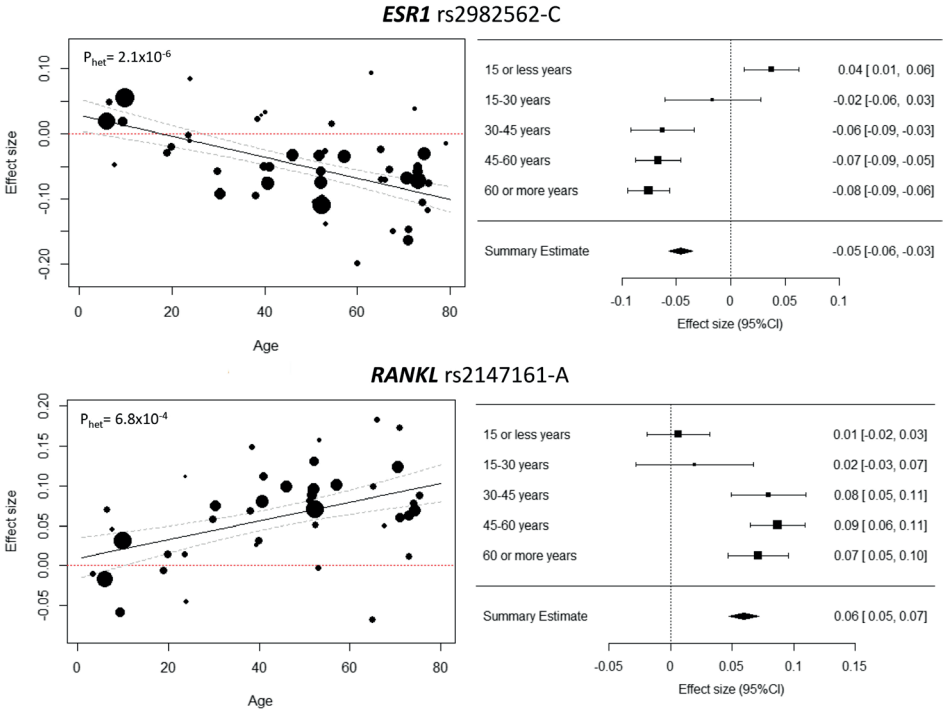


Figure 2 | Age Dependence of the Genetic Variant Effect in the Meta-regression. The panels display leading SNPs from two loci exhibiting significant evidence for age influences. Heterogeneity p values (phet) are reported for the overall meta-analysis. In the left panels, each circle represents a study subgroup (i.e., study divided in age strata), with the circle size proportional to the inverse variance of the SNP main effect. In the right panels, forest plots display estimates obtained from each age-bin meta-analysis, with the symbol size proportional to the inverse variance of the SNP main effect.

Shared Genetic Architecture of TB-BMD, Fracture, and Other Traits

SNP heritability of TB-BMD in the European samples was estimated to be 0.259 (SE 0.017). TB-BMD was highly genetically correlated with BMD measured at other skeletal sites ($r>0.9$). Among the non-BMD traits, all types of fracture showed the highest correlation ($r = 0.61$ [$p = 1.6x10^{-27}$]). The MR approach indicated a strong causal relation where, per 1 standard deviation decrease in genetically determined TB-BMD, there is 56% increase in the risk of fracture (odds ratio 1.56 [1.50–1.62]). Other anthropometric, metabolic, and disease traits showed significant (yet weak) correlation with TB-BMD (**Supplementary table 9, Figure 3**). In contrast, other established risk factors for osteoporosis such as menopause or age of menarche showed no significant genetic correlation with TB-BMD.

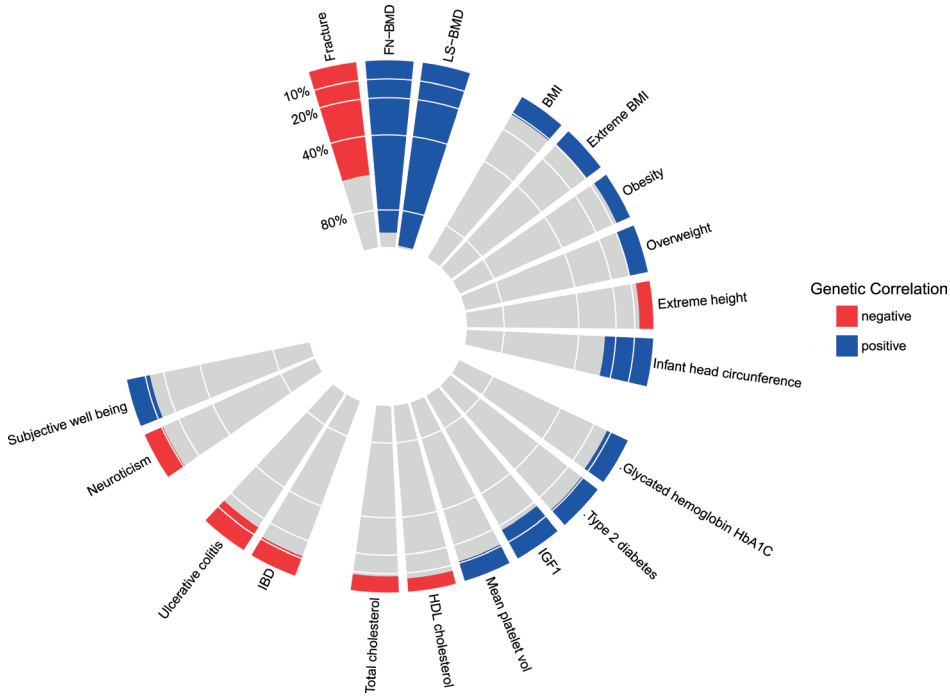


Figure 3 | Genetic Correlations between TB-BMD and Other Traits and Diseases. Calculation was based on the summary statistics of the only-European meta-analysis (N = 56,284) and estimated by LD score regression implemented in LDHub. The diagram shows only traits whose correlation with TB-BMD was significant ($p < 0.05$).

Biological and Functional Knowledge of the Genes in BMD-Associated Loci

Loci not previously reported and their potential role in bone metabolism are summarized in Table 1. Several loci harbor genes implicated directly in bone metabolism (*SLC8A1* [MIM: 182305], *PLCL1* [MIM: 600597], *ADAMTS5* [MIM: 605007]), affecting osteoblast or osteoclast differentiation and activity (*CSF1* [MIM: 120420], *DUSP5* [MIM: 603069], S*MAD3* [MIM: 603109], *SMAD9* [MIM: 603295], *CD44* [MIM: 107269]), participating in Wnt signaling (FZD7 [MIM: 603410], *TCF7L1* [MIM: 604652]), or regulating processes such as manganese or calcium absorption (*GCKR* [MIM: 600842], *DGKD* [MIM: 601826], *SLC30A10* [MIM: 611146]) among others;[39–60] while genes in at least 14 loci exert a potential novel role in bone biology. Rodent knockout models of several genes in the implicated loci show an altered skeletal phenotype (e.g., ostoepetrosis [Csf139], increased bone resorption [Aqp1,[48] Cyp19a1,[55] Cd4451], impaired skeletogenesis [Apc, 47 Runx1,[59] Smad3[56]], deformities in the axial skeleton [Btg1, [61] Atpaf262]). An effect on bone can be inferred for genes in other associated loci, for example, *CYP19A1* (MIM: 107910) in 15q21.2 is an estrogen synthesis gene,

estrogen being a key compound for bone maturation and maintenance, and *ZKSCAN5* (MIM: 611272) in 7q22.1 is associated with circulating dehydroepiandrosterone sulfate (DHEAS) levels.[49] DHEAS levels are positively correlated with BMD in adults and post-menopausal women.[63] Across these loci, not previously reported as associated with BMD variation, we identified six exonic variants associated with TB-BMD, three of which were nonsynonymous variants all cataloged as benign both by SIFT and PolyPhen-2. We also identified 53 GWS coding variants in known loci, of which 33 are non-synonymous (**Supplementary Table 10**). Only a low frequency variant in *LRP5* (MIM: 603506), rs4988321/A (11:68174189, MAF = 0.04), has a clinical annotation, constituting a homozygous G-to-A transition variant identified in a person with osteoporosis-pseudoglioma syndrome (*OPPG* [MIM: 259770]).[64]

DEPICT Analyses

Based on the overall meta-analysis, 53 genes were prioritized (FDR < 0.05), 15 of them mapping to loci not previously described (**Supplementary Table 11**). Cells and tissues from the musculoskeletal system presented the largest enrichment of gene expression within the associated loci (**Figure 4**). These genes were overrepresented in 182 pathways clustered in 25 ''meta gene-sets'' (**Supplementary Table 12**). The large majority of the clusters are involved in musculoskeletal development and bone homeostasis (**Figure 4**). The most significant of these implicated the regulation of cell growth and the TGFB signaling pathway and its mediating SMAD proteins. Restricting the DEPICT analysis to the subset of not previously reported associated regions resulted in significant enrichment of genes expressed in the musculoskeletal and immunological systems (**Supplementary Figure 8**). Genes mapping to these loci were overrepresented in the SMAD binding pathway and TGFBR2 PPI (protein-protein interaction) subnetwork (FDR < 0.05).

Functional Annotation to MicroRNA Binding Sites

We then assessed whether the index SNPs of the 80 GWS loci detected in the main and subsequent GWAS (or their proxies in strong LD; r2 > 0.8) were located in predicted mi-croRNA binding sites within the genes' 3' UTRs and thus were expected to disrupt the regulation of gene expression (**Supplementary Table 13**). The index SNP within the 3' UTR of *ZKSCAN5* (mapping to a locus not previously identified), rs34670419 (MAF = 0.04), is predicted to create a binding site for miR-382-3p, a microRNA that is expressed in osteocytes and has been recently shown to be involved in osteogenic differentiation.65 In addition, eight proxy SNPs (mapping to *PSMD13*, *ABCF2*, *GALNT3*, *PKDCC*, *REEP5*, *PPP6R3*, *AAGAB*, and *TOM1L2*) are predicted to influence the binding of microRNAs to transcripts of their host gene.

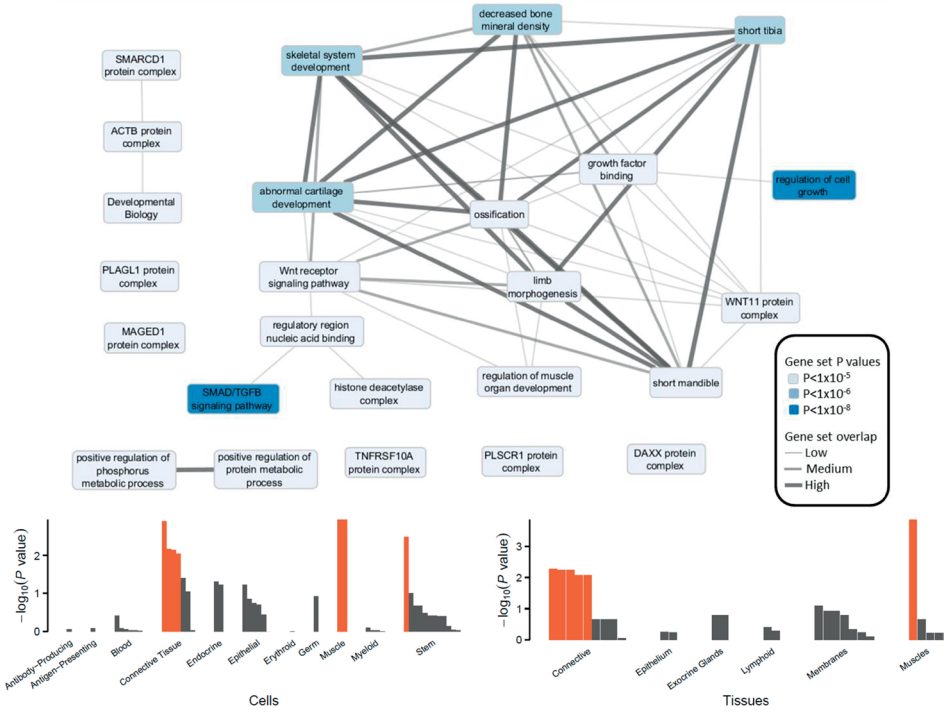


Figure 4 | Results for Gene Set and Cell/Tissue Enrichment Analyses
Top: 25 Meta gene sets were defined from similarity clustering of significantly enriched gene sets (FDR < 5%). Each meta gene set was named after one of its member gene sets. The color of the meta gene sets represents the p value of the member set. Interconnection line width represents the Pearson correlation ρ between the gene membership scores for each meta gene set (ρ < 0.3, no line; 0.3 ≤ ρ < 0.5, narrow width; 0.5 ≤ ρ < 0.7, medium width; ρ ≥ 0.7, thick width). Bottom: Bars represent the level of evidence for genes in the associated loci to be expressed in any of the 209 medical subject heading (MeSH) tissue and cell type annotations. Highlighted in orange are these cell/tissue types significantly (FDR < 5%) enriched for the expression of the genes in the associated loci.

Functional Enrichment Analysis of Trait-Associated Variants

As typically found in GWASs, the great majority of identified associations emerged from non-coding common variants and hold no direct annotation to molecular mechanisms. To assess whether there is relative enrichment of regulatory genomic marks underlying the associated variants in a cell-specific context, we used GARFIELD.[32] We found relative ubiquitous enrichment for TB-BMD variants (empirical $p < 2.4x10^{-4}$) in DNase I hypersensitive sites across the different cell types (**Figure S9**). Further, we found higher levels of fold-enrichment for enhancers (median 3.6, range [2.7, 4.4]) and promotors (median 3.2, range [2.9, 3.5]) than for transcribed regions (median 1.8, range [1.5, 2.2]).

Gene Expression in Bone Cells and Knockout Animal Models

From the 53 genes prioritized by DEPICT, only 49 [20] had mouse ortholog (**Table S14**). From these genes, only *Mepe* (osteocyte-specific) and *Foxl1* were not expressed in murine osteoblast or osteoclast. Moreover, 61% of the prioritized genes were expressed in human cells *in vitro* during osteoblast or osteoclast differentiation (**Table S14**). *AQP1* was the only prioritized gene mapping to a locus not previously reported showing no expression in the human bone cells differentiation experiments.

Knockout models were widely available in at least one of the different databases assessed. Nevertheless, in-depth bone phenotyping performed under the OBCD project was available for only four knockout models (**Table S15**). Two of these, *DUSP5* and *CD300LG*, showed no significant bone phenotype. The *TCF7L1* knockout model only showed lower cortical diameter in the femur without other clear bone phenotype. Nevertheless, *TCF7L1* was shown to be expressed during osteoblastogenesis. Conversely, homozygous knockout for *CREB3L1* showed a clear bone phenotype consisting of low BMC both at the vertebrae and femur together with a strong trabecular and cortical phenotype affecting bone strength (**Figure S10**). *CREB3L1* maps to 11p11.2, a previously identified BMD locus5 harboring *ARHGAP1* and *LRP4* as candidates to underlie the GWAS signal in a region of extended LD.

DISCUSSION

This meta-analysis of TB-BMD comprising up to 66,000 individuals identified variants in 36 loci not previously reported and replicated at GWS level several association signals identified by GWASs of diverse bone phenotypes. Bioinformatics analyses suggest enrichment of these 36 loci for genes expressed in the musculoskeletal system and sol-idly represented in the SMAD binding pathway and the TGFBR2 PPI subnetwork. We also demonstrate that for variants in few loci the size of the effect is age dependent; variants in two loci (*RIN3* and *TSHZ3*) were identified only by the age-stratified analyses despite less power (smaller sample size); while for variants in two other loci (*ESR1* and *RANKL*) there was significant evidence of age heterogeneity derived from a meta-regression of the genetic effects with age. Our results strengthen the evidence that genetic variants influence BMD from a young age and support the value of peak bone mass as an important determinant of bone health later in life.

Traditionally, DXA-BMD measurements performed at sites of high fracture risk (i.e., femoral neck, lumbar spine and forearm) have been used in genetic epidemiological investigations of bone health in adults. Instead, we have used BMD measurements derived from total body scans. Not only do we show a high overlap of association signals with previous GWASs of different bone traits, including DXA, pQCT, and ultrasound

measurements, but we have also identified unreported loci. Five known associations failed to replicate in our studies, even though we cannot discard these associations as false positives, because these results might also indicate that variants whose effect is highly specific to skeletal sites, skeletal properties, sex, or age groups cannot be detected in our TB-BMD meta-analysis. It is plausible that more variants of this type exist and will be discovered as site-specific BMD meta-analyses are performed in increasingly powered set-tings. Furthermore, the genetic correlation of TB-BMD with BMD measured at other sites was close to one. Nevertheless, we found that a decrease of one standard deviation in the genetically determined TB-BMD resulted in at least 50% higher odds of suffering a fracture. Significant genetic correlations with other traits (i.e., BMI, IGF1, and ulcerative colitis) reflect the systemic context of skeletal biology and merit further study by future efforts to elucidate the underlying mechanisms.

Genes in the associated loci were highly expressed in the musculoskeletal system and overrepresented in gene sets related to bone development. The prioritized gene *CREB3L1* (MIM: 616215) in 11p11.2 observed a clear bone phenotype in our mouse knockout model, which corroborates the findings of previous work showing substantial rescue of *CREB3L1* deficiency with bisphosphonates and its critical role for bone formation.[66] This locus, characterized by extended LD, also harbors *LRP4* (MIM: 604270) whose knockout model presents with increased trabecular and cortical bone mass.[67] This is in line with our conditional analysis identifying multiple independent signals in the region, making it likely that both genes are influencing bone biology. Altogether, we demonstrated that TB-BMD offers a powerful alternative to identify genetic variants associated with bone metabolism.

Variants mapping to 14q32 harboring *RIN3* (MIM: 610223) were associated only at a GWS level in children (i.e., <15 years) and were only nominally significant in the elderly group (i.e., >60 years). This age-related heterogeneity may explain why this locus has not been detected in BMD meta-analyses in adults, although being identified in relation to pediatric BMD*7* and Paget disease (PDB [MIM: 602080]) GWASs.[68] In addition, another signal mapping to 19q12 harboring T*SHZ3* (MIM: 614119) was significant in adults aged 45–60 years but not in other age groups analyzed or in previous studies, alluding to a false-positive association, so replication of this finding is necessary.

Our analyses revealed variants in the 6q25.1 (*ESR1*) and 13q14.11 (*RANKL*) loci demonstrating the most compelling evidence for age-modulation effects. The 6q25.1 locus harboring *ESR1* (MIM: 133430), an important genetic factor in normal BMD variability, was not associated with BMD in children below 15 years of age, where the largest cohorts (i.e., Avon Longitudinal Study of Parents and Children [ALSPAC] and the Generation R Study) comprise predominantly pre-pubertal children. As levels

of estradiol before puberty are low,[69] a negligible effect of *ESR1* variants on BMD is expected. Likewise, in mouse models the expression of *RANKL* (MIM: 602642) in bone is markedly increased with advancing age from young to adult and related to bone loss.[70] Accordingly, variants influencing *RANKL* expression show a larger effect later in life. In general, a substantial heterogeneity of the genetic effects in the overall meta-analysis was explained by age, but the inclusion of larger sample sizes (avoiding age exclusion criteria and incrementing statistical power) leveled off the loss of power due to the heterogeneity of the genetic effects.

In brief, variants with evidence of age-specific effects were exceptional in our study. These results might reflect a lack of statistical power as only SNPs showing suggestive evidence ($p < 5x10^{-6}$) of association with TB-BMD in the overall meta-analysis were tested for age-specific effects. This selection criteria aimed to include SNPs whose heterogeneity might have hampered their statistical significance in the overall meta-analysis, and at the same time maximize the power to discover variants with real age-dependent effects. Alternatively, these results indicate that most of the genetic variants identified so far, by us and others, influence BMD from early ages onward, and their effect persist throughout the life course. However, variants in 27 of the 42 loci (64%) showing nominal evidence for age-dependent effects had larger effects in the older groups. Nonetheless, this requires careful interpretation given the uneven sample sizes be-tween the age groups and the criteria to select markers for the meta-regression based on significance in the overall meta-analysis. Collectively, this argues in favor of enlarging studies focused on younger populations-where the statistical power is still restricted-to discover additional genetic variants influencing BMD.

Our study has some limitations. A key disadvantage of our design is that we group the data based on age spans rather than life stages. Crucial information for this assessment, such as puberty onset in children and adolescents or menopausal status in the adults, was not available across the majority of the cohorts. Other strategies like using smaller age spans will result in even less statistical power of the discovery setting. Similarly, despite the large sample size of our study, we identified very few variants in the low-frequency spectrum (MAF < 5%), indicating that comprehensive surveys of rare variation influencing BMD still require even larger sample sizes, on top of better resources for imputation of the rarer variants, possibly needing population-specific references. Such strategies will be key to explain a larger fraction of the genetic variability of BMD phenotypes, as illustrated for other traits such as height or BMI.[71] Moreover, the identified SNPs are, in the vast majority, non-coding variants, raising the possibility that the causal genes are different from the candidate genes we have prioritized based on the current biological knowledge and bioinformatic prediction tools. Additional functional studies are required to determine the potential role of the genes in the identified loci.

In conclusion, we performed a genome-wide survey for association with DXA-derived TB-BMD, combining data from five age groups including children and older individuals. In contrast to previous large-scale meta-analyses,[5,6] we used DXA-derived TB-BMD rather than measurements on specific skeletal sites prone to fracture to identify genetic factors influencing BMD variation. We demonstrate that TB-BMD is a valid phenotype for this purpose, as we replicated more than 90% of the previously reported signals. Most importantly, we identify variants in 36 loci associated with TB-BMD not previously reported by previous GWASs of bone phenotypes. Our results show steadiness in the magnitude of the genetic effects on BMD for most of the BMD-associated variants. While the contrasting skeletal physiology across different age periods is well established (i.e., endochondral ossification, linear growth, modeling, remodeling, etc.), peak bone mass acquisition remains the major determinant of variability at any age. These findings strongly support the importance of the bone accrual process in the definition of BMD status and fracture susceptibility throughout the life course.

Detailed acknowledgments and online resources can be found in the published article online

https://www.sciencedirect.com/science/article/pii/S0002929717304949?via %3Dihub

REFERENCES

1. Johnell, O., Kanis, J.A., Oden, A., Johansson, H., De Laet, C., Delmas, P., Eisman, J.A., Fujiwara, S., Kroger, H., Mellstrom, The American Journal of Human Genetics 102, 88–102, January 4, 2018 99 D., et al. (2005). Predictive value of BMD for hip and other fractures. J. Bone Miner. Res. 20, 1185–1194.
2. Farr, J.N., and Khosla, S. (2015). Skeletal changes through the lifespan–from growth to senescence. Nat. Rev. Endocrinol. 11, 513–521.
3. Hendrickx, G., Boudin, E., and Van Hul, W. (2015). A look behind the scenes: the risk and pathogenesis of primary osteoporosis. Nat. Rev. Rheumatol. 11, 462–474.
4. Tenne, M., McGuigan, F., Besjakov, J., Gerdhem, P., and A˚ kesson, K. (2013). Degenerative changes at the lumbar spine– implications for bone mineral density measurement in elderly women. Osteoporos. Int. 24, 1419–1428.
5. Estrada, K., Styrkarsdottir, U., Evangelou, E., Hsu, Y.H., Duncan, E.L., Ntzani, E.E., Oei, L., Albagha, O.M., Amin, N., Kemp, J.P., et al. (2012). Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat. Genet. 44, 491–501.
6. Zheng, H.F., Forgetta, V., Hsu, Y.H., Estrada, K., Rosello-Diez, A., Leo, P.J., Dahia, C.L., Park-Min, K.H., Tobias, J.H., Kooperberg, C., et al.; AOGC Consortium; and UK10K Consortium (2015). Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature 526, 112–117.
7. Kemp, J.P., Medina-Gomez, C., Estrada, K., St Pourcain, B., Heppe, D.H., Warrington, N.M., Oei, L., Ring, S.M., Kruithof, C.J., Timpson, N.J., et al. (2014). Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. PLoS Genet. 10, e1004423.
8. Paternoster, L., Lorentzon, M., Lehtima¨ki, T., Eriksson, J., Ka¨ho¨nen, M., Raitakari, O., Laaksonen, M., Sieva¨nen, H., Viikari, J., Lyytika¨inen, L.P., et al. (2013). Genetic determinants of trabecular and cortical volumetric bone mineral densities and bone microstructure. PLoS Genet. 9, e1003247.
9. Moayyeri, A., Hsu, Y.H., Karasik, D., Estrada, K., Xiao, S.M., Nielson, C., Srikanth, P., Giroux, S., Wilson, S.G., Zheng, H.F., et al. (2014). Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum. Mol. Genet. 23, 3054–3068.
10. Medina-Gomez, C., Kemp, J.P., Estrada, K., Eriksson, J., Liu, J., Reppe, S., Evans, D.M., Heppe, D.H., Vandenput, L., Herrera, L., et al. (2012). Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet. 8, e1002718.
11. Yang, T.L., Guo, Y., Liu, Y.J., Shen, H., Liu, Y.Z., Lei, S.F., Li, J., Tian, Q., and Deng, H.W. (2012). Genetic variants in the SOX6 gene are associated with bone mineral density in both Caucasian and Chinese populations. Osteoporos. Int. 23, 781–787.
12. Zhang, L., Choi, H.J., Estrada, K., Leo, P.J., Li, J., Pei, Y.F., Zhang, Y., Lin, Y., Shen, H., Liu, Y.Z., et al. (2014). Multistage genome-wide association meta-analyses identified two new loci for bone mineral density. Hum. Mol. Genet. 23, 1923– 1933.
13. Pei, Y.F., Xie, Z.G., Wang, X.Y., Hu, W.Z., Li, L.B., Ran, S., Lin, Y., Hai, R., Shen, H., Tian, Q., et al. (2016). Association of 3q13.32 variants with hip trochanter and intertrochanter

bone mineral density identified by a genome-wide association study. Osteoporos. Int. 27, 3343–3354.

14. Styrkarsdottir, U., Thorleifsson, G., Eiriksdottir, B., Gudjonsson, S.A., Ingvarsson, T., Center, J.R., Nguyen, T.V., Eisman, J.A., Christiansen, C., Thorsteinsdottir, U., et al. (2016). Two Rare Mutations in the COL1A2 Gene Associate With Low Bone Mineral Density and Fractures in Iceland. J. Bone Miner. Res. 31, 173–179.
15. Koller, D.L., Zheng, H.F., Karasik, D., Yerges-Armstrong, L., Liu, C.T., McGuigan, F., Kemp, J.P., Giroux, S., Lai, D., Edenberg, H.J., et al. (2013). Meta-analysis of genome-wide studies identifies WNT16 and ESR1 SNPs associated with bone mineral density in premenopausal women. J. Bone Miner. Res. 28, 547–558.
16. Nielson, C.M., Liu, C.T., Smith, A.V., Ackert-Bicknell, C.L., Reppe, S., Jakobsdottir, J., Wassel, C., Register, T.C., Oei, L., Alonso, N., et al. (2016). Novel Genetic Variants Associated With Increased Vertebral Volumetric BMD, Reduced Vertebral Fracture Risk, and Increased Expression of SLC1A3 and EPHB2. J. Bone Miner. Res. 31, 2085–2097.
17. Styrkarsdottir, U., Thorleifsson, G., Gudjonsson, S.A., Sigurdsson, A., Center, J.R., Lee, S.H., Nguyen, T.V., Kwok, T.C., Lee, J.S., Ho, S.C., et al. (2016). Sequence variants in the PTCH1 gene associate with spine bone mineral density and osteoporotic fractures. Nat. Commun. 7, 10129.
18. Winkler, T.W., Day, F.R., Croteau-Chonka, D.C., Wood, A.R., Locke, A.E., Ma¨gi, R., Ferreira, T., Fall, T., Graff, M., Justice, A.E., et al.; Genetic Investigation of Anthropometric Traits (GIANT) Consortium (2014). Quality control and conduct of genome-wide association meta-analyses. Nat. Protoc. 9, 1192–1212.
19. Simino, J., Shi, G., Bis, J.C., Chasman, D.I., Ehret, G.B., Gu, X., Guo, X., Hwang, S.J., Sijbrands, E., Smith, A.V., et al.; LifeLines Cohort Study (2014). Gene-age interactions in blood pressure regulation: a large-scale investigation with the CHARGE, Global BPgen, and ICBP Consortia. Am. J. Hum. Genet. 95, 24–38.
20. Viechtbauer, W. (2010). Conducting Meta-Analyses in R with the metafor Package. J. Stat. Softw. 36, 1–48.
21. Winkler, T.W., Kutalik, Z., Gorski, M., Lottaz, C., Kronenberg, F., and Heid, I.M. (2015). EasyStrata: evaluation and visualization of stratified genome-wide association meta-analysis data. Bioinformatics 31, 259–261.
22. Yang, J., Ferreira, T., Morris, A.P., Medland, S.E., Madden, P.A.F., Heath, A.C., Martin, N.G., Montgomery, G.W., Weedon, M.N., Loos, R.J., et al.; Genetic Investigation of ANthropometric Traits (GIANT) Consortium; and DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium (2012). Conditional and joint multiple-SNP analysis of GWAS summary statistics identifies additional variants influencing complex traits. Nat. Genet. 44, 369–375, S1–S3.
23. Bulik-Sullivan, B.K., Loh, P.R., Finucane, H.K., Ripke, S., Yang, J., Patterson, N., Daly, M.J., Price, A.L., Neale, B.M.; and Schizophrenia Working Group of the Psychiatric Genomics Consortium (2015). LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. Nat. Genet. 47, 291–295.
24. Bulik-Sullivan, B., Finucane, H.K., Anttila, V., Gusev, A., Day, F.R., Loh, P.R., Duncan, L., Perry, J.R., Patterson, N., Robinson, E.B., et al.; ReproGen Consortium; Psychiatric Genomics Consortium; and Genetic Consortium for Anorexia Nervosa of the Wellcome Trust Case Control Consortium 3 (2015). An atlas of genetic correlations across human diseases and traits. Nat. Genet. 47, 1236–1241.

25. Zheng, J., Erzurumluoglu, A.M., Elsworth, B.L., Kemp, J.P., Howe, L., Haycock, P.C., Hemani, G., Tansey, K., Laurin, C., Pourcain, B.S., et al.; Early Genetics and Lifecourse 100 The American Journal of Human Genetics 102, 88–102, January 4, 2018 Epidemiology (EAGLE) Eczema Consortium (2017). LD Hub: a centralized database and web interface to perform LD score regression that maximizes the potential of summary level GWAS data for SNP heritability and genetic correlation analysis. Bioinformatics 33, 272–279.
26. Burgess, S., Butterworth, A., and Thompson, S.G. (2013). Mendelian randomization analysis with multiple genetic variants using summarized data. Genet. Epidemiol. 37, 658–665.
27. Pers, T.H., Karjalainen, J.M., Chan, Y., Westra, H.J., Wood, A.R., Yang, J., Lui, J.C., Vedantam, S., Gustafsson, S., Esko, T., et al.; Genetic Investigation of ANthropometric Traits (GIANT) Consortium (2015). Biological interpretation of genome-wide association studies using predicted gene functions. Nat. Commun. 6, 5890.
28. Bhattacharya, A., Ziebarth, J.D., and Cui, Y. (2014). PolymiRTS Database 3.0: linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. Nucleic Acids Res. 42, D86–D91.
29. Gong, J., Tong, Y., Zhang, H.M., Wang, K., Hu, T., Shan, G., Sun, J., and Guo, A.Y. (2012). Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. Hum. Mutat. 33, 254–263.
30. Barenboim, M., Zoltick, B.J., Guo, Y., and Weinberger, D.R. (2010). MicroSNiPer: a web tool for prediction of SNP effects on putative microRNA targets. Hum. Mutat. 31, 1223–1232.
31. Ghanbari, M., Franco, O.H., de Looper, H.W., Hofman, A., Erkeland, S.J., and Dehghan, A. (2015). Genetic Variations in MicroRNA-Binding Sites Affect MicroRNA-Mediated Regulation of Several Genes Associated With Cardio-metabolic Phenotypes. Circ Cardiovasc Genet 8, 473–486.
32. Iotchkova, V., Ritchie, G.R.S., Geihs, M., Morganella, S., Min, J.L., Walter, K., Timpson, N.J., Dunham, I., Birney, E., and Soranzo, N. (2016). GARFIELD - GWAS Analysis of Regulatory or Functional Information Enrichment with LD correction. bioRxiv. https://doi.org/10.1101/085738.
33. Dickinson, M.E., Flenniken, A.M., Ji, X., Teboul, L., Wong, M.D., White, J.K., Meehan, T.F., Weninger, W.J., Westerberg, H., Adissu, H., et al.; International Mouse Phenotyping Consortium; Jackson Laboratory; Infrastructure Nationale PHENOMIN, Institut Clinique de la Souris (ICS); Charles River Laboratories; MRC Harwell; Toronto Centre for Phenogenomics; Wellcome Trust Sanger Institute; and RIKEN BioResource Center (2016). High-throughput discovery of novel developmental phenotypes. Nature 537, 508–514.
34. de Angelis, M.H., Nicholson, G., Selloum, M., White, J., Morgan, H., Ramirez-Solis, R., Sorg, T., Wells, S., Fuchs, H., Fray, M., et al.; EUMODIC Consortium (2015). Analysis of mammalian gene function through broad-based phenotypic screens across a consortium of mouse clinics. Nat. Genet. 47, 969–978.
35. Freudenthal, B., Logan, J., Croucher, P.I., Williams, G.R., Bassett, J.H.; and Sanger Institute Mouse Pipelines (2016). Rapid phenotyping of knockout mice to identify genetic determinants of bone strength. J. Endocrinol. 231, R31–R46.
36. Kim, K., Punj, V., Kim, J.M., Lee, S., Ulmer, T.S., Lu, W., Rice, J.C., and An, W. (2016). MMP-9 facilitates selective proteolysis of the histone H3 tail at genes necessary for proficient osteoclastogenesis. Genes Dev. 30, 208–219.
37. van de Peppel, J., Strini, T., Tilburg, J., Westerhoff, H., van Wijnen, A.J., and van Leeuwen, J.P. (2017). Identification of Three Early Phases of Cell-Fate Determination during Osteogenic

and Adipogenic Differentiation by Transcription Factor Dynamics. Stem Cell Reports 8, 947–960.

38. Koek, W.N.H., van der Eerden, B.C.J., Alves, R.D.A.M., van Driel, M., Schreuders-Koedam, M., Zillikens, M.C., and van Leeuwen, J.P.T.M. (2017). Osteoclastogenic capacity of peripheral blood mononuclear cells is not different between women with and without osteoporosis. Bone 95, 108–114.
39. Dobbins, D.E., Sood, R., Hashiramoto, A., Hansen, C.T.,Wilder, R.L., and Remmers, E.F. (2002). Mutation of macrophage colony stimulating factor (Csf1) causes osteopetrosis in the tl rat. Biochem. Biophys. Res. Commun. 294, 1114–1120.
40. Claro da Silva, T., Hiller, C., Gai, Z., and Kullak-Ublick, G.A. (2016). Vitamin D3 transactivates the zinc and manganese transporter SLC30A10 via the Vitamin D receptor. J. Steroid Biochem. Mol. Biol. 163, 77–87.
41. O'Seaghdha, C.M., Wu, H., Yang, Q., Kapur, K., Guessous, I., Zuber, A.M., Ko¨ttgen, A., Stoudmann, C., Teumer, A., Kutalik, Z., et al.; SUNLIGHT Consortium; and GEFOS Consortium (2013). Meta-analysis of genome-wide association studies identifies six new Loci for serum calcium concentrations. PLoS Genet. 9, e1003796.
42. Speliotes, E.K., Yerges-Armstrong, L.M., Wu, J., Hernaez, R., Kim, L.J., Palmer, C.D., Gudnason, V., Eiriksdottir, G., Garcia, M.E., Launer, L.J., et al.; NASH CRN; GIANT Consortium; MAGIC Investigators; and GOLD Consortium (2011). Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet. 7, e1001324.
43. Ousingsawat, J., Wanitchakool, P., Schreiber, R., Wuelling, M., Vortkamp, A., and Kunzelmann, K. (2015). Anoctamin-6 controls bone mineralization by activating the calcium transporter NCX1. J. Biol. Chem. 290, 6270–6280.
44. Shy, B.R., Wu, C.I., Khramtsova, G.F., Zhang, J.Y., Olopade, O.I., Goss, K.H., and Merrill, B.J. (2013). Regulation of Tcf7l1 DNA binding and protein stability as principal mechanisms of Wnt/b-catenin signaling. Cell Rep. 4, 1–9.
45. Tsutsumi, K., Matsuda, M., Kotani, M., Mizokami, A., Murakami, A., Takahashi, I., Terada, Y., Kanematsu, T., Fukami, K., Takenawa, T., et al. (2011). Involvement of PRIP, phospholipase C-related, but catalytically inactive protein, in bone formation. J. Biol. Chem. 286, 31032–31042.
46. Li, Y., and Dudley, A.T. (2009). Noncanonical frizzled signaling regulates cell polarity of growth plate chondrocytes. Development 136, 1083–1092.
47. Miclea, R.L., Karperien, M., Bosch, C.A., van der Horst, G., van der Valk, M.A., Kobayashi, T., Kronenberg, H.M., Rawadi, G., Akc¸akaya, P., Lo¨wik, C.W., et al. (2009). Adenomatous polyposis coli-mediated control of beta-catenin is essential for both chondrogenic and osteogenic differentiation of skeletal precursors. BMC Dev. Biol. 9, 26.
48. Wu, Q.T., Ma, Q.J., He, C.Y., Wang, C.X., Gao, S., Hou, X., and Ma, T.H. (2007). Reduced bone mineral density and bone metabolism in aquaporin-1 knockout mice. Chem. Res. Chin. Univ. 23, 297–299.
49. Zhai, G., Teumer, A., Stolk, L., Perry, J.R.B., Vandenput, L., Coviello, A.D., Koster, A., Bell, J.T., Bhasin, S., Eriksson, J., et al.; MuTHER Consortium (2011). Eight common genetic variants associated with serum DHEAS levels suggest a key role in ageing mechanisms. PLoS Genet. 7, e1002025.
50. Moon, S.J., Lim, M.A., Park, J.S., Byun, J.K., Kim, S.M., Park, M.K., Kim, E.K., Moon, Y.M., Min, J.K., Ahn, S.M., et al. The American Journal of Human Genetics 102, 88–102, January 4, 2018

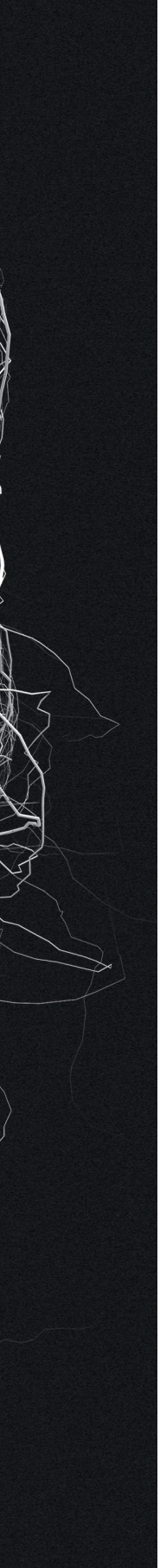
101 (2014). Dual-specificity phosphatase 5 attenuates autoimmune arthritis in mice via reciprocal regulation of the Th17/ Treg cell balance and inhibition of osteoclastogenesis. Arthritis Rheumatol. 66, 3083–3095.

51. Hayer, S., Steiner, G., Go¨rtz, B., Reiter, E., Tohidast-Akrad, M., Amling, M., Hoffmann, O., Redlich, K., Zwerina, J., Skriner, K., et al. (2005). CD44 is a determinant of inflammatory bone loss. J. Exp. Med. 201, 903–914.
52. Krishnan, V., Bryant, H.U., and Macdougald, O.A. (2006). Regulation of bone mass by Wnt signaling. J. Clin. Invest. 116, 1202–1209.
53. Ryan, Z.C., Craig, T.A., Filoteo, A.G., Westendorf, J.J., Cartwright, E.J., Neyses, L., Strehler, E.E., and Kumar, R. (2015). Deletion of the intestinal plasma membrane calcium pump, isoform 1, Atp2b1, in mice is associated with decreased bone mineral density and impaired responsiveness to 1, 25-dihydroxyvitamin D3. Biochem. Biophys. Res. Commun. 467, 152–156.
54. Tsukamoto, S., Mizuta, T., Fujimoto, M., Ohte, S., Osawa, K., Miyamoto, A., Yoneyama, K., Murata, E., Machiya, A., Jimi, E., et al. (2014). Smad9 is a new type of transcriptional regulator in bone morphogenetic protein signaling. Sci. Rep. 4, 7596.
55. Miyaura, C., Toda, K., Inada, M., Ohshiba, T., Matsumoto, C., Okada, T., Ito, M., Shizuta, Y., and Ito, A. (2001). Sex- and agerelated response to aromatase deficiency in bone. Biochem. Biophys. Res. Commun. 280, 1062–1068.
56. Borton, A.J., Frederick, J.P., Datto, M.B., Wang, X.F., and Weinstein, R.S. (2001). The loss of Smad3 results in a lower rate of bone formation and osteopenia through dysregulation of osteoblast differentiation and apoptosis. J. Bone Miner. Res. 16, 1754–1764.
57. Kim, K., Kim, J.H., Lee, J., Jin, H.M., Kook, H., Kim, K.K., Lee, S.Y., and Kim, N. (2007). MafB negatively regulates RANKLmediated osteoclast differentiation. Blood 109, 3253–3259.
58. Tamamura, Y., Otani, T., Kanatani, N., Koyama, E., Kitagaki, J., Komori, T., Yamada, Y., Costantini, F., Wakisaka, S., Pacifici, M., et al. (2005). Developmental regulation of Wnt/ beta-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. J. Biol. Chem. 280, 19185–19195.
59. Soung, Y., Kalinowski, J., Baniwal, S.K., Jacome-Galarza, C.E., Frenkel, B., Lorenzo, J., and Drissi, H. (2014). Runx1-mediated regulation of osteoclast differentiation and function. Mol. Endocrinol. 28, 546–553.
60. Li, V., Raouf, A., Kitching, R., and Seth, A. (2004). Ets2 transcription factor inhibits mineralization and affects target gene expression during osteoblast maturation. In Vivo 18, 517–524.
61. Tijchon, E., van Ingen Schenau, D., van Opzeeland, F., Tirone, F., Hoogerbrugge, P.M., Van Leeuwen, F.N., and Scheijen, B. (2015). Targeted Deletion of Btg1 and Btg2 Results in Homeotic Transformation of the Axial Skeleton. PLoS ONE 10, e0131481.
62. Koscielny, G., Yaikhom, G., Iyer, V., Meehan, T.F., Morgan, H., Atienza-Herrero, J., Blake, A., Chen, C.K., Easty, R., Di Fenza, A., et al. (2014). The International Mouse Phenotyping Consortium Web Portal, a unified point of access for knockout mice and related phenotyping data. Nucleic Acids Res. 42, D802–D809.
63. Ghebre, M.A., Hart, D.J., Hakim, A.J., Kato, B.S., Thompson, V., Arden, N.K., Spector, T.D., and Zhai, G. (2011). Association between DHEAS and bone loss in postmenopausal women: a 15-year longitudinal population-based study. Calcif. Tissue Int. 89, 295–302.
64. Gong, Y., Slee, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A.M., Wang, H., Cundy, T., Glorieux, F.H., Lev, D., et al.; Osteoporosis-Pseudoglioma Syndrome Collaborative Group

(2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107, 513–523.

65. Heilmeier, U., Hackl, M., Skalicky, S., Weilner, S., Schroeder, F., Vierlinger, K., Patsch, J.M., Baum, T., Oberbauer, E., Lobach, I., et al. (2016). Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells In Vitro. J. Bone Miner. Res. 31, 2173–2192.
66. Sekiya, H., Murakami, T., Saito, A., Hino, S., Tsumagari, K., Ochiai, K., and Imaizumi, K. (2010). Effects of the bisphosphonate risedronate on osteopenia in OASIS-deficient mice. J. Bone Miner. Metab. 28, 384–394.
67. Boudin, E., Yorgan, T., Fijalkowski, I., Sonntag, S., Steenackers, E., Hendrickx, G., Peeters, S., De Mare´, A., Vervaet, B., Verhulst, A., et al. (2017). The Lrp4 R1170Q homozygous knock-in mouse recapitulates the bone phenotype of sclerosteosis in humans. J. Bone Miner. Res. 32, 1739–1749.
68. Albagha, O.M., Wani, S.E., Visconti, M.R., Alonso, N., Goodman, K., Brandi, M.L., Cundy, T., Chung, P.Y., Dargie, R., Devogelaer, J.P., et al.; Genetic Determinants of Paget's Disease (GDPD) Consortium (2011). Genome-wide association identifies three new susceptibility loci for Paget's disease of bone. Nat. Genet. 43, 685–689.
69. Courant, F., Aksglaede, L., Antignac, J.P., Monteau, F., Sorensen, K., Andersson, A.M., Skakkebaek, N.E., Juul, A., and Bizec, B.L. (2010). Assessment of circulating sex steroid levels in prepubertal and pubertal boys and girls by a novel ultrasensitive gas chromatography-tandem mass spectrometry method. J. Clin. Endocrinol. Metab. 95, 82–92.
70. Cao, J., Venton, L., Sakata, T., and Halloran, B.P. (2003). Expression of RANKL and OPG correlates with age-related bone loss in male C57BL/6 mice. J. Bone Miner. Res. 18, 270–277.
71. Yang, J., Bakshi, A., Zhu, Z., Hemani, G., Vinkhuyzen, A.A., Lee, S.H., Robinson, M.R., Perry, J.R., Nolte, I.M., van Vliet-Ostaptchouk, J.V., et al.; LifeLines Cohort Study (2015). Genetic variance estimation with imputed variants finds negligible missing heritability for human height and body mass index. Nat. Genet. 47, 1114–1120.





2.2.4

Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study

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**Denotes equal supervision

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ABSTRACT

Objective: To identify the genetic determinants of fracture risk and assess the role of 15 clinical risk factors on osteoporotic fracture risk. Design: Meta-analysis of genome wide association studies (GWAS) and a two-sample mendelian randomisation approach.

Setting: 25 cohorts from Europe, United States, east Asia, and Australia with genome wide genotyping and fracture data. Participants: A discovery set of 37 857 fracture cases and 227 116 controls; with replication in up to 147 200 fracture cases and 150 085 controls. Fracture cases were defined as individuals (>18 years old) who had fractures at any skeletal site confirmed by medical, radiological, or questionnaire reports. Instrumental variable analyses were performed to estimate effects of 15 selected clinical risk factors for fracture in a two-sample mendelian randomisation framework, using the largest previously published GWAS meta-analysis of each risk factor.

Results: Of 15 fracture associated loci identified, all were also associated with bone mineral density and mapped to genes clustering in pathways known to be critical to bone biology (e.g., *SOST*, *WNT16*, and *ESR1*) or novel pathways (*FAM210A*, *GRB10,* and *ETS2*). Mendelian randomisation analyses showed a clear effect of bone mineral density on fracture risk. One standard deviation decrease in genetically determined bone mineral density of the femoral neck was associated with a 55% increase in fracture risk (odds ratio 1.55 (95% confidence interval 1.48 to 1.63; $P=1.5\times10^{-68}$). Hand grip strength was inversely associated with fracture risk, but this result was not significant after multiple testing correction. The remaining clinical risk factors (including vitamin D levels) showed no evidence for an effect on fracture.

Conclusions: This large scale GWAS meta-analysis for fracture identified 15 genetic determinants of fracture, all of which also influenced bone mineral density. Among the clinical risk factors for fracture assessed, only bone mineral density showed a major causal effect on fracture. Genetic predisposition to lower levels of vitamin D and estimated calcium intake from dairy sources were not associated with fracture risk.

INTRODUCTION

The United Nations recently predicted that the ratio of people aged 65 years and older to those aged 15-64 years will triple globally by 2100.[1] Musculoskeletal conditions are the most common causes of severe pain and physical disability, and their prevalence will increase with the ageing of society.[2] One of the largest musculoskeletal burdens is attributable to osteoporotic fractures, the incidence of which increases exponentially with age.[3] Therefore, the prevention of fractures is an important public health goal. The causes of multifactorial common diseases, such as osteoporotic fractures, include genetic and environmental influences, as well as their interactions (gene by environment, or G×E). Clinically useful risk factors for the prediction of osteoporotic fracture risk need not be necessarily causal and have been implemented by well validated risk score algorithms such as FRAX[4] and the Garvan[5,6] fracture risk calculator. Yet, the extent to which modification of predictive clinical risk factors reduces fracture risk is not generally known. A better understanding of causal mechanisms will enable prevention strategies, direct the launch of proper clinical trials, and provide targets for effective lifestyle and pharmacological interventions. Acquiring this knowledge is particularly timely and relevant considering the increasing recognition that many individuals at high fracture risk often do not receive fracture prevention interventions.[7] Fracture risk is a moderately heritable trait (whereby h2 is roughly 30%),[8,9] for which no large scale, genome wide association studies (GWAS) have been undertaken so far. Large GWAS meta-analyses can also be used to perform mendelian randomisation analyses to explore the causal effects of heritable risk factors on disease in people, while reducing bias due to confounding (because genetic variation is essentially randomly assigned at conception) or reverse causation (because allele assignment always precedes disease onset).[10] Conceptually similar to a randomised controlled trial, the mendelian randomisation approach enables an assessment of the cumulative effect of a genetically determined exposure on fracture risk, minimising the biases that frequently weaken observational studies.

Understanding whether interventions aimed at clinical risk factors would reduce fracture risk is important, because clinicians often ensure that such risk factors are optimised in individuals at high risk of fracture. If the risk factors are not causal, then such optimisation would not decrease fracture risk. Therefore, to better understand genetic and clinical risk factors for fracture, we undertook a large scale GWAS for fracture risk in up to 264 973 participants (37 857 fracture cases) in the discovery stage and in conjunction with the largest available GWAS for clinical risk factors, determined the genetic correlation (shared heritability) of key clinical risk factors and fracture. We then performed mendelian randomisation studies to explore the causal effect of these risk factors on fracture.

SUBJECTS AND METHODS

Study Populations

A total of 23 cohorts with genome wide genotyping and fracture data were recruited globally through the GEnetic Factors for OSteoporosis consortium (GEFOS; http://www.gefos.org/). These cohorts were predominantly of European descent and from Europe (n=13), North America (n=8), Australia (n=1), and east Asia (n=1; **Supplementary Tables 1A and 2A**), and included 20 439 fracture cases and 78 843 controls. After meta-analysis, replication of promising findings was performed initially in the GENOMOS consortium (18 779 cases and 32 078 controls from 29 additional studies, **Supplementary Tables 1B and 2B**). Two additional large GWAS (UK Biobank, 14 492 cases and 130 563 controls; EPIC-Norfolk study, 2926 cases and 17 710 controls) were then included in the discovery set, comprising in total 37 857 cases and 227 116 controls (aged 18-106 years, including 69% women). Genetic markers reaching genome wide significance in this expanded meta-analysis and previously reported bone mineral density markers associated with fracture[11] were additionally replicated in 147 200 cases and 150 085 controls from 23andMe, a personal genetic company (23andMe GWAS participants were customers who consented to participate in research with self reported fracture data). **Supplementary Figure 1** shows the overall study design. To enable two-sample mendelian randomisation studies, we compiled summary level results from the largest available GWAS meta-analyses performed so far on a large set of clinical risk factors for fracture (**Table 1**). All studies were approved by their respective institutional ethics review committees and all participants provided written informed consent.

Study endpoint (fracture definition)

To maximise the statistical power to detect genetic loci, we used an inclusive definition of fracture, which was successfully used in previous efforts to test bone mineral density associated variants for association with fracture[11,27] and allowed us to undertake the largest GWAS on fracture risk so far.

Fracture cases were defined as those individuals (>18 years old) who had fractures at any skeletal site confirmed by medical, radiological, or questionnaire reports (table S3). Fractures of the fingers, toes, and skull as well as high trauma fractures were excluded whenever possible, although there have been some reports that even high trauma fractures are also predicted by low bone mineral density and are predictive of future low trauma fracture. [28,29] Controls were defined as individuals (>18 years old) from the same cohorts, without a history of fracture.

Table 1 | Fracture risk factors assessed and number of samples in each genome wide association study

Disease or Trait	Total Sample Size
Femoral neck bone mineral density[11]	32,961
Lumbar spine bone mineral density[11]	31,800
Age at menopause[12]	69,360
Rheumatoid arthritis[13]	58,284 (14,361 cases)
Inflammatory bowel disease[14]	34,652 (12,882 cases)
Type 1 diabetes[15]	26,890 (9,934 cases)
Thyroid stimulating hormone[16]	26,523
Homocysteine[17]	44,147
Grip strength[18]	142,035
Age of puberty[19]	182,416
Fasting glucose[20.21]	58,074
Coronary heart disease[22]	107,432 (41,513 cases)
Type 2 diabetes[23]	56,862 (12,171 cases)
Vitamin D levels[24.25]	33,996
Dairy calcium intake[26*]	171,213†

Lactase intolerance (MCM6-rs4988235) was used as a proxy for dairy consumption
† Effect estimates were derived from ref 26.

Fracture GWAS Meta-analysis and Replication

Genome wide genotyping was performed in each cohort by use of Illumina or Affymetrix genome wide genotyping chips (**Supplementary table 4A**) and was imputed to ensure accurate ascertainment of nearly all common genetic variation above a minor allele frequency threshold of 1%. After strict quality control criteria were applied to samples and single nucleotide polymorphisms (SNPs), we followed a consortium wide standardised analytical plan to assess the association of SNPs with risk of fracture. We used logistic regression adjusted for sex, age (simple and quadratic terms), height, and weight, testing additive (per allele) genetic effects. Before performing meta-analysis, three separate meta-analytical centres checked the data independently. All individual GWAS were corrected by genomic control before we performed a fixed effects meta-analysis using METAL software. A total of 2 539 801 autosomal SNPs present in more than two studies were meta-analysed. We took forward for replication a set of promising SNPs for de novo genotyping in 26 studies at LGC Genomics (UK), using KASP genotyping as described previously[27] (**Supplementary table 4B**) and tested them in three more studies (**Supplementary table 4C**), for a total of 29 studies. Allele and genotype frequencies of all genotyped variants followed Hardy-Weinberg equilibrium proportions. To obtain unbiased estimates of effect size, all SNPs associated at a genome wide significant level (that is, $P<5\times10^{-8}$) and previously known

bone mineral density fracture loci[11] were tested for replication in the 23andMe cohort (**Supplementary table 4C**).

Genetic Determinants of Risk Factors for Fracture

We used the genetic determinants of 15 available clinical risk factors from the largest GWAS datasets available. Genome wide association analyses have been published for bone mineral density (femoral neck and lumbar spine),[11] age of puberty,[19] age at menopause,[12] grip strength,[18] vitamin D,[24 25] homocysteine,[17] thyroid stimulating hormone level,[16] fasting glucose,[20 21] type 1 diabetes,[15] type 2 diabetes,[23] rheumatoid arthritis,[13] inflammatory bowel disease,[14] and coronary artery disease.[22] The well established lactose intolerance marker (LCT(C/T- 13910) polymorphism; rs4988235)[30] was used as a surrogate to assess long term differences in dairy derived calcium intake.[31] Additional risk factors were considered for inclusion; however, at the time of analyses, well powered GWAS were not available for some risk factors of interest including alcohol intake,[32] smoking[34] and plasma calcium levels.[35] Body mass index[36] was not evaluated given that the fracture discovery analysis was adjusted for body weight and height.

Genetic Correlation

We used LD score regression to estimate the genetic correlation of the selected clinical risk factors and fracture (table 1).[37] This method estimates the degree of shared genetic risk factors between two diseases or traits, and was applied to 11 of the 15 selected risk factors for fracture (since genome wide association results were not publicly available for type 1 diabetes and thyroid stimulating hormone and dairy calcium intake). We accounted for multiple testing by using a conservative Bonferroni correction for 12 tests (that is, $\alpha=4.2\times10^{-3}$). We also tested whether the above mentioned risk factors were genetically correlated with bone mineral density.

Mendelian Randomisation

Next, we undertook mendelian randomisation analyses to estimate effects of 15 selected clinical risk factors in two-sample mendelian randomisation framework. The mendelian randomisation approach was based on the following assumptions:

a) The genetic variants used as instrumental variables are associated with the clinical risk factors.
b) The genetic variants are not associated with any confounders of the exposure-outcome relation.
c) The genetic variants are associated with fracture only through the clinical risk factors-that is, a lack of pleiotropy (**Figure 1**).

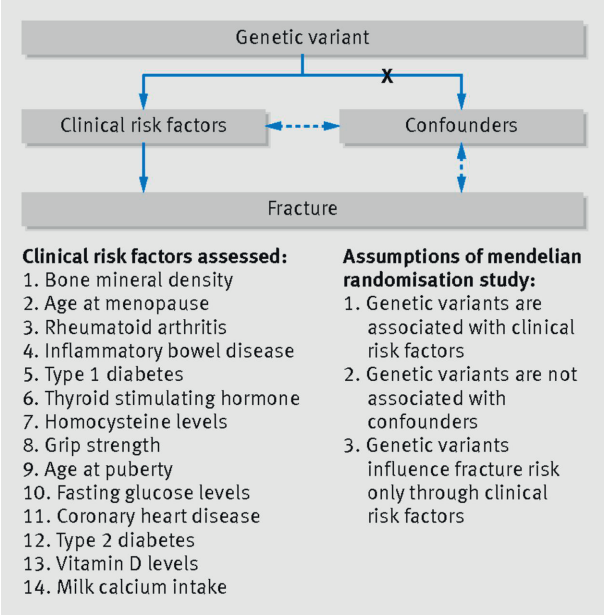


Figure 1 | Mendelian randomization study design

We used the largest previously published GWAS meta-analyses of the risk factors, at the time of analyses, to maximise statistical power (**Supplementary table 5A**) . [38-41] To reduce potential bias due to population stratification, we restricted the analyses to studies with participants of European descent. To ensure independence between the SNPs used to evaluate the association of the risk factor and fracture risk, we grouped by LD (r2>0.05) those SNPs achieving genome wide significance, keeping only the SNP with the lowest P value per group. Next, we recorded the effect size and standard error attributed to each allele's effect on the risk factor (**Supplementary table 5B**). Finally, for age of menopause, we performed sex specific mendelian randomisation analysis in women only.

The resulting individual SNP effect estimates were pooled by use of the Wald type ratio estimator, which is formally analogous to an inverse weighted meta-analysis.[42] Again, we applied a conservative Bonferroni corrected threshold (that is, $\alpha=3.3\times10^{-3}$, because 15 risk factors were assessed) to account for the multiple risk factors tested. We also tested the assumptions underlying the mendelian randomisation approach (**Figure 1**). To test the third assumption (a lack of pleiotropic effects of the SNPs on the outcome, independent of the exposure), we used mendelian randomisation-Egger regression.[43] Moreover, as sensitivity analyses for robust causal inference, we additionally performed mendelian randomisation analyses using a weighted median estimator and penalised weighted median estimator. We also tested the effect of the same clinical risk factors on bone mineral density[27] using the same methods. For the

binary exposures, the odds ratios were converted (by multiplying log-odds ratios by 0.693 and then exponentiating) in order to represent the odds ratio per doubling of the odds of susceptibility to disease.[44] Finally, we undertook mendelian randomisation power calculations[45] for all such analyses.

RESULTS

Genetic Loci Associated with Fracture

We saw was no evidence of excessive genomic inflation (λ=1.02, LD score intercept=0.99) in the GWAS meta-analysis, suggesting that the results were not biased because of population stratification, genotyping artefacts, or cryptic family relationships (**Figure 2**). As shown in **Table 2 and Figure 2**, 15 genomic loci were associated at a genome wide significant level with fracture risk after meta-analysis of the discovery (table S6A) and replication (Supplementary tables 6B, 6C, and 6D) stages. All loci were at, or near, loci previously shown to be associated with bone mineral density,[11,27,46-52] a major determinant of fracture risk (**Supplementary Table 6E and Supplementary Figure 2**). The effect sizes of these common SNPs on fracture risk was modest (odds ratios ranging from 1.03 to 1.10), which is consistent with GWAS findings for other complex diseases.[53]

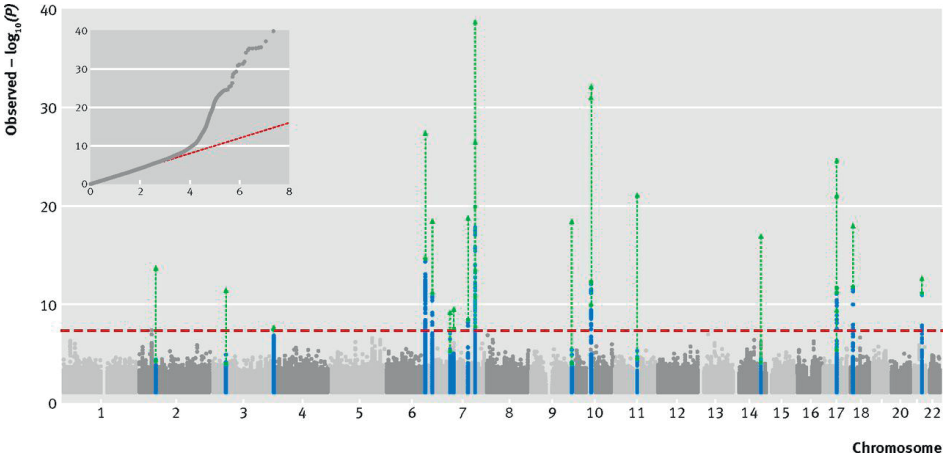


Figure 2 | Manhattan plot of -log10 association P values for discovery meta-analysis, and quantile-quantile plot (QQ plot) of the distribution of observed -log10 association P values against the expected null distribution for discovery meta-analysis. Dashed horizontal red line=genome wide significant (GWS) threshold ($P<5\times10^{-8}$); blue dots=SNPs at GWS loci that are within 500kb of leading SNPs in previous genome wide association studies with different bone traits. Green lines and triangles=combined −log10 association P values after replication in the 23andMe cohort

Table 2 | Fracture risk factors assessed and number of samples in each genome wide association study

Locus	Candidate gene	SNP	Distance to gene (kb)	EA	EAF	Discovery stage*		Replication stage*		Combined*			
						Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	No of fractures	I^2
2p16.2	*SPTBN1*	rs4233949	-23.21	G	0.61	1.03 (1.02-1.05)	6.9×10^{-5}	1.04 (1.05-1.05)	8.9×10^{-11}	1.03 (1.02-1.04)	2.8×10^{-14}	185,057	22.4
3p22.1	*CTNNB1*	rs430727	107.2	T	0.45	1.03 (1.02-1.05)	1.0×10^{-4}	1.03 (1.02-1.04)	1.1×10^{-8}	1.03 (1.02-1.04)	5.0×10^{-12}	185,057	0
6q22.33	*RSPO3*	rs10457487	0	C	0.51	1.06 (1.05-1.08)	2.3×10^{-15}	1.04 (1.03-1.05)	1.7×10^{-15}	1.05 (1.04-1.06)	4.8×10^{-28}	185,057	5
6q25.1	*ESR1*	rs2982570	0	C	0.58	1.05 (1.04-1.07)	8.1×10^{-12}	1.03 (1.02-1.04)	5.2×10^{-10}	1.04 (1.03-1.05)	4.5×10^{-19}	185,057	23
7q31.31	*WNT16, CPED1*	rs2908007	-3.25, 24.27	A	0.6	1.08 (1.06-1.10)	1.2×10^{-20}	1.05 (1.04-1.06)	5.6×10^{-22}	1.06 (1.05-1.07)	2.3×10^{-39}	185,055	0
7q21.3	*C7orf76, SHFM1*	rs6465508	0,0	G	0.34	1.05 (1.03-1.07)	4.0×10^{-9}	1.04 (1.03-1.05)	4.1×10^{-12}	1.04 (1.03-1.05)	2.0×10^{-19}	185,056	35
7p14.1	*STARD3NL*	rs6959212	-89.01	T	0.34	1.04 (1.02-1.06)	6.9×10^{-6}	1.02 (1.01-1.04)	1.1×10^{-5}	1.03 (1.02-1.04)	8.8×10^{-10}	185,057	15.6
7p12.1	*GRB10, COBL*	rs1548607	40.33, -182.4	G	0.32	1.05 (1.03-1.07)	3.2×10^{-8}	1.02 (1.01-1.04)	2.1×10^{-4}	1.03 (1.02-1.05)	4.7×10^{-10}	185,052	40
9q34.11	*FUBP3*	rs7851693	0	G	0.35	1.03 (1.01-1.06)	1.3×10^{-4}	1.05 (1.06-1.06)	4.8×10^{-16}	1.04 (1.03-1.05)	5.0×10^{-19}	185,057	23.5
10q21.1	*MBL2, DKK1*	rs11003047	-90.63	G	0.11	1.09 (1.07-1.12)	6.2×10^{-12}	1.08 (1.07-1.10)	1.4×10^{-21}	1.09 (1.07-1.10)	9.5×10^{-33}	185,057	0
11q13.2	*LRP5*	rs3736228	0	T	0.15	1.05 (1.03-1.07)	3.0×10^{-5}	1.07 (1.05-1.08)	2.8×10^{-18}	1.06 (1.05-1.08)	1.0×10^{-21}	185,056	24.6
14q32.12	*RPS6KA5*	rs1286083	0	T	0.82	1.04 (1.02-1.06)	8.8×10^{-5}	1.05 (1.04-1.07)	3.0×10^{-14}	1.05 (1.04-1.06)	1.6×10^{-17}	185,085	43.3

Table 2 | Fracture risk factors assessed and number of samples in each genome wide association study (*continued*)

Locus	Candidate gene	SNP	Distance to gene (kb)	EA	EAF	Discovery stage*		Replication stage*		Combined*			
						Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	No of fractures	I^2
17q21.31	*SOST, DUSP3, MEOX1*	rs2741856	-4.26, -16.65 88.02	G	0.92	1.11 (1.08-1.14)	2.4×10^{-12}	1.08 (1.06-1.11)	5.3×10^{-15}	1.10 (1.07-1.11)	3.1×10^{-25}	184,977	0
18p11.21	*FAM210A, RNMT*	rs4635400	0, -7.15	A	0.36	1.06 (1.04-1.07)	1.5×10^{-12}	1.03 (1.02-1.04)	2.7×10^{-9}	1.04 (1.03-1.05)	1.1×10^{-18}	185,057	22
21q22.2	*ETS2*	rs9980072	141.9	G	0.73	1.06 (1.04-1.08)	8.4×10^{-12}	1.03 (1.01-1.04)	1.8×10^{-5}	1.04 (1.03-1.05)	3.4×10^{-13}	185,057	36

EA=effect allele; EAF=effect allele frequency; I^2=index of heterogeneity.

* Discovery stage (37,857 cases; 227,116 controls); replication stage (147,200 cases; 150,085 controls); combined (185,057 cases; 377,201 controls).

Genetic Correlations with Clinical Risk Factors

SNPs influencing bone mineral density were strongly and inversely correlated with odds of fracture (**Table 3**; genetic correlation -0.59, $P=2\times10^{-24}$ for femoral neck bone mineral density, with similar results for lumbar spine bone mineral density, -0.53, $P=1\times10^{-20}$). By contrast, none of the remaining clinical risk factors evaluated was strongly genetically correlated with risk of fracture with the exception of homocysteine (**Table 3**). Genetically increased risk of type 2 diabetes was positively correlated with femoral neck bone mineral density, while genetically increased grip strength had positive correlations with bone mineral density of both the femoral neck and lumbar spine (**Supplementary Table 7**).

Table 3 | Estimated genetic correlation between fracture and other clinical risk factors

Disease or trait	Genetic correlation (95%CI)	P
Femoral neck bone mineral density	-0.59 (-0.70, -0.48)	2×10^{-24}
Lumbar spine bone mineral density	-0.53 (-0.64, -0.42)	1×10^{-20}
Age at menopause	-0.12 (-0.23, -0.003)	0.04
Rheumatoid arthritis	0.02 (-0.10, 0.14)	0.74
Inflammatory bowel disease	-0.01 (-0.13, 0.11)	0.90
Homocysteine levels	0.22 (0.07, 0.37)	0.004
Grip strength	-0.10 (-0.21, 0.01)	0.07
Age of puberty	0.03 (-0.05, 0.11)	0.43
Fasting glucose	-0.05 (-0.19, 0.09)	0.46
Coronary heart disease	-0.05 (-0.09, 0.19)	0.48
Type 2 diabetes	-0.07 (-0.22, 0.08)	0.35
Vitamin D levels	0.23 (-0.52, 0.98)	0.56

Mendelian Randomisation

Using mendelian randomisation analyses to assess the effect of the 15 risk factors on fracture, we saw evidence for a major effect of genetically decreased bone mineral density on fracture risk (**Figure 3** and **Table 4**; odds ratio per standard deviation decrease in femoral neck bone mineral density=1.55, 95% confidence interval 1.48 to 1.63, $P=1.5\times10^{-68}$). We also observed a large effect of grip strength on fracture risk (2.14, 1.13 to 4.04, P=0.01), but these results had wide confidence intervals and were not significant after multiple testing correction. Vitamin D levels assessed by use of 25-hydroxyvitamin D variants were not found to be linearly associated with increased fracture risk (odds ratio per standard deviation decrease=0.84, 95% confidence interval 0.70 to 1.02, P=0.07). Most of these mendelian randomisation effects did not seem to be strongly influenced by directional pleiotropy, because the

intercepts of the mendelian randomisation-Egger test were tightly centred around the null, except for rheumatoid arthritis, type 2 diabetes, grip strength, glucose, and homocysteine levels (**Table 4**). The estimates from the inverse variance weighted fixed meta-analysis were very similar to the estimates from the weighted median and penalised weighted median method (**Supplementary Table 8**). However, despite some indication of causality of fasting glucose levels on fracture risk (**Supplementary Table 8**) in the median weighted analyses, it did not surpass the multiple testing threshold.

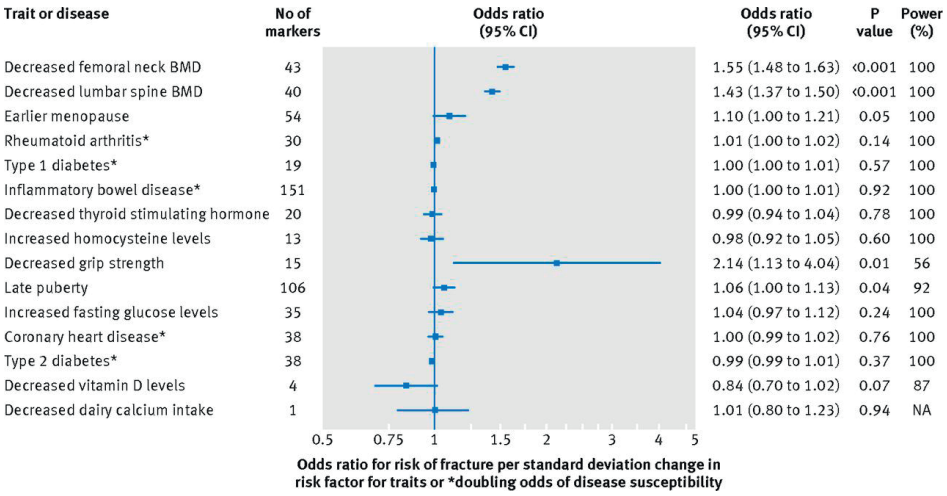


Figure 3 | Forest plot showing effect of 15 genetically determined risk factors on fracture risk. Power=statistical power to detect an odds ratio of 1.15 at $\alpha \leq 3.3 \times 10^{-3}$; NA=not applicable; BMD=bone mineral density

Consistent with the results of the genetic correlation analyses, we found that none of the other evaluated clinical risk factors had evidence of a causal effect on risk of fracture, despite adequate statistical power (mean=98% (range 56-100%), **Table 4**).[45]When evaluating the effect of genetically increased risk factors on bone mineral density (**Supplementary table 9**), only age of puberty had an effect on bone mineral density after accounting for multiple testing; fasting glucose, type 2 diabetes, and age at menopause had marginal effects, consistent with a recent mendelian randomisation study of type 2 diabetes and glycaemic traits on bone mineral density.[54]

We next undertook careful evaluation of the three mendelian randomisation assumptions. The first assumption was verified by the selection of only common variants (minor allele frequency >5%) strongly associated with the clinical risk factor ($P<5 \times 10^{-8}$). After performing a thorough literature search, we can exclude reported associations between the genetic variants and potential confounding factors (second assumption). Finally, by using mendelian randomisation-Egger regression, we found

no evidence of the presence of pleiotropy between the instruments and the outcomes (**Table 4**).

Table 4 | Estimated effects of 15 genetically determined risk factors on fracture risk

		Inverse variance weighted meta-analysis			Mendelian randomisation-Egger regression§	
Trait or disease	**No of markers**	**Odds ratio (95% CI)***	**P**	**Power (%)‡**	**Intercept (95% CI)**	**P**
Decreased femoral neck BMD	43	1.55 (1.48-1.63)	1.5×10^{-68}	100	-0.001 (-0.011, 0.008)	0.83
Decreased lumbar spine BMD	40	1.43 (1.37-1.50)	2.3×10^{-55}	100	0.005 (-0.006, 0.014)	0.93
Earlier menopause	54	1.10 (1.00-1.21)	0.05	100	0.001 (-0.006, 0.007)	0.83
Rheumatoid arthritis†	30	1.01 (1.00-1.02)	0.14	100	0.010 (0.003, 0.017)	0.005
Type 1 diabetes†	19	1.00 (1.00-1.01)	0.57	100	0.003 (-0.004, 0.010)	0.39
Inflammatory bowel disease†	151	1.00 (1.00-1.01)	0.92	100	0.000 (-0.003, 0.004)	0.86
Decreased thyroid stimulating hormone	20	0.99 (0.94-1.04)	0.78	100	0.005 (-0.019, 0.009)	0.47
Increased homocysteine levels	13	0.98 (0.92-1.05)	0.60	100	0.013 (0.001, 0.026)	0.03
Decreased grip strength	15	2.14 (1.13-4.04)	0.01	56	0.107 (0.011, 0.203)	0.03
Late puberty	106	1.06 (1.00-1.13)	0.04	92	0.004 (-0.002, 0.009)	0.21
Increased fasting glucose levels	35	1.04 (0.97-1.12)	0.24	100	-0.008 (-0.014, -0.002)	0.01
Coronary heart disease†	38	1.00 (0.99-1.02)	0.76	100	0.003 (-0.007, 0.013)	0.57
Type 2 diabetes†	38	0.99 (0.99-1.01)	0.37	100	-0.009 (-0.016, -0.002)	0.02
Decreased vitamin D levels	4	0.84 (0.70-1.02)	0.07	87	-0.014 (-0.103, 0.074)	0.56
Decreased dairy calcium intake	1	1.01 (0.80-1.23)	0.94	NA	NA	NA

NA=not applicable; BMD=bone mineral density.

* Odds ratio is for the risk of fracture per standard deviation change in the risk factor for traits (1 standard deviation change=0.13 g femoral neck BMD, 0.18 g lumbar spine BMD, 3.9 years earlier menopause, 0.76 mIU/L thyroid stimulating hormone, 11.3 kg grip strength, 1.42 years late puberty, 0.62 mmol/L fasting glucose, 25.2 nmol/L vitamin D), or †risk of fracture per doubling of odds of disease susceptibility; dairy calcium intake units are servings/day. Estimates obtained using a fixed effects model.

‡ Statistical power to detect an odds ratio of 1.15 at $\alpha \leq 3.3\times10-3$.

§ Egger regression analyses can be performed if the number of genetic variants is more than two; Egger effect estimates are presented in table S7.

¶ Findings that remain associated (that is, $\alpha<3.3\times10-3$) after correction for multiple testing.

DISCUSSION

Principal Findings and Interpretation

In this large GWAS for fracture, we identified genetic determinants (at 15 loci) of fracture and tested the role of 15 selected clinical risk factors on fracture risk and bone mineral density. Using mendelian randomisation analyses, we demonstrated that genetically decreased bone mineral density (and, to a lesser extent, hand grip strength) was the only clinical risk factor among those tested, with evidence for an effect on fracture risk. By contrast, despite high statistical power, none of the other tested and well accepted risk factors (eg, rheumatoid arthritis and other causes of secondary osteoporosis) or any of the other clinically relevant risk factors (vitamin D levels, dairy food derived calcium intake, fasting glucose, type 2 diabetes, and coronary heart disease) had evidence of a major causal effect on fracture risk. Furthermore, all identified genetic determinants of fracture also influenced bone mineral density. In our previous work,[11] we tested 96 bone mineral density markers for association with fracture. In the meta-analysis, 14 bone mineral density loci were associated with fracture risk ($P<5\times10^{-4}$), of which six surpassed genome-wide significance ($P<5\times10^{-8}$). In our current project, we began with GWAS meta-analysis for fracture risk. We confirmed the 2p16.2 (*SPTBN1*), 7q21.3 (*SHFM1*), 10q21.1 (*MBL2/DKK1*), 11q13.2 (*LRP5*), and 18p11.21 (*FAM210A*) loci, and observed an increased signal at *SOST, CPED1/WNT16, FUPB3, DCDC5, RPS6KA5, STARD3NL*, and *CTNNB1*. Lastly, we added the 6q22.33 (*RSPO3*), 6q25.1 (*ESR1*), 7p12.1 (*GRB10/COBL*), and 21q22.2 (*ETS2*) loci to the list of novel fracture loci. Among the genome wide significant loci associated with fracture, several contain well established causal proteins for fracture risk that are targets for clinically useful osteoporotic fracture treatments, such as *ESR1*, which encodes the oestrogen receptor, and *SOST*, which encodes sclerostin.[55] These discoveries highlight known and novel factors in pathways critical to bone biology (that is, Wnt, for mesenchymal stem cell differentiation) as well as potential new factors and biological pathways that might constitute future drug targets.[56]

All the discovered fracture loci are also associated with bone mineral density, implying that skeletal fragility characterised by reduced bone mineral density is central to the pathophysiology of osteoporotic fracture. This contention is in line with the significant genetic correlation we identified between bone mineral density and fracture. Our mendelian randomisation analyses also indicate that the suggestive effect of late puberty and earlier age at menopause on fracture risk is, at least partly, mediated through reduced bone mineral density. By contrast, hand grip strength was not found to be a determinant of bone mineral density, and vice versa. Still, grip strength could be a proxy for overall muscle strength and risk of falling, and could be involved in a pathway leading to fracture independently of bone mineral density.[56]

However, the hand grip estimates holds wide confidence intervals in our analyses expressed in standard deviations to allow comparison to other risk factors. We believe that these large standard deviations can be attributed to multiple factors, including effort and encouragement of the participants, posture, position, and intrinsic measurement variability between individuals. A recent effort using UK Biobank data also showed through mendelian randomisation that higher grip strength is associated with decreased fracture risk.[18] As such, inclusion of grip strength (or a different assessment of muscle function such as leg strength) could improve the predictive performance of risk prediction calculators that already contain bone mineral density, just as has been reported for a history of falls.[56]

Older individuals at high risk of fractures often have low levels of vitamin D (owing to low dietary intake and sun exposure). Therefore, fracture prevention guidelines have suggested the use of vitamin D supplementation in the general population.[57,58] These recommendations have contributed to the marked increase in vitamin D use in older populations worldwide, where in the United States alone the proportion of individuals aged 70 years and older who use at least 1000 IU of vitamin D daily increased about 100-fold from 2000 to 2014.[59] Despite these guideline recommendations, it is unclear whether modestly low levels of vitamin D, rather than profoundly low values, are causally associated with a higher risk of fracture. Our mendelian randomisation work examined a linear relation between vitamin D levels and fracture risk. We did not test for the possibility of a threshold dependent relation-that is, effects that could be present only at very low levels of vitamin D. Nevertheless, our analyses showed that vitamin D levels had no protective linear effect on fracture in community dwelling individuals, despite adequate statistical power. We also show, in line with other previous reports,[60-62] no evidence for a causal effect of vitamin D levels on bone mineral density. Although a threshold effect is likely to be present, where profoundly lowered vitamin D levels do increase risk of fracture, our mendelian randomisation results strongly suggest that increasing levels of vitamin D in the (non-deficient) general population is unlikely to decrease risk of fracture. Likewise, calcium supplementation has been called into question recently. A mendelian randomisation study found that higher levels of serum calcium are a risk factor for coronary heart disease,[63] supporting the current recommendation of not exceeding total calcium intake of 1200 mg/day in older individuals.[64] Further, our study assessed the lactose persistence variant as a surrogate of long term intake of dairy calcium (used previously as an instrument for dairy consumption in association with blood pressure[26] and other traits), and found no evidence for a protective effect of sustained intake of dairy derived calcium on fracture risk.

Comparison with Other Studies

Previous observational studies and clinical trials have reported the beneficial effect of vitamin D[65,66] or calcium[67] supplementation on fracture risk reduction, findings which are not supported by our results. These discrepant findings can be due to inadequate methods or high heterogeneity induced, for example, by combining community dwelling participants and inpatients in the same analysis. Consistent with our findings, a recent meta-analysis of 33 randomised trials[68] (n=51 145) found that supplementation with calcium, vitamin D, or both did not decrease the incidence of fractures in community dwelling older adults. Findings such as these should be interpreted with caution, because they do not necessarily apply to patients undergoing osteoporosis treatment, considering that trials evaluating osteoporosis treatment are carried out concomitant with vitamin D and calcium supplementation.

Studies seeking to show whether these supplements do increase the efficacy of osteoporotic treatment or decrease adverse events (that is, hypocalcaemia) are lacking. In either case, screening for vitamin D deficiency and seeking its correction should be warranted before the initiation of anti-resorptive treatment. Moreover, in a recent mendelian randomisation study investigating the role of 25-hydroxy-vitamin D in maintaining bone mineral density,[62] increased levels of 25-hydroxy-vitamin D had no effect on bone mineral density measured by dual energy x-ray absorptiometry (n=32 965; 0.02 g/ cm^2 change in femoral neck bone mineral density per standard deviation increase in 25-hydroxy-vitamin D). However, increased 25-hydroxy-vitamin D was associated with a slight reduction in heel bone mineral density estimated by ultrasonography (n=142 487; -0.03 g/cm^2 change in estimated bone mineral density per standard deviation increase in 25-hydroxy-vitamin D). These results are consistent with our mendelian randomisation findings of no causal effect of vitamin D levels on fracture.

Implications for Clinicians

Our mendelian randomisation findings are relevant to clinical care. Although clinical risk factors, when used jointly in well validated prediction algorithms, predict fracture risk, our findings are a reminder that clinically relevant changes in most of these risk factors are unlikely to result in large differences in fracture risk. These findings also suggest clinical outcomes such as fracture risk can be subject to bias owing to uncontrolled confounding in observational epidemiological studies. A strength of our study design is that mendelian randomisation limits this potential confounding, because alleles are essentially assigned randomly at conception, and are therefore not generally affected by confounders. Furthermore, because allele assignment must precede fracture, mendelian randomisation is not prone to bias due to reverse causation. These

findings provide guidance for the design of future clinical trials on interventions that are more likely to be successful in reducing fracture risk.

Epidemiological studies have shown that older people with a fracture will have abnormal bone mineral density in the osteoporotic range (that is, T score lower than -2.5 standard deviations), but most will have a fracture will be osteopenic (T score between -1 and -2.5 standard deviations). In fact, about 87% of women and 82% of men with a non-vertebral fracture have a T score lower than -1.0.[69] Our findings suggest that low bone mineral density (not only after reaching the osteoporotic range) constitutes a risk factor that captures a substantial and causal part of the influences that increase risk for all types of fracture. Therefore, interventions targeting an increase in bone mineral density (presuming this is associated with improvements in bone structure or quality) are likely to have pivotal roles in reducing fracture risk.

The interpretation of our findings merits careful consideration for some of the risk factors. Hyperthyroidism is an established risk factor for fracture, and we have not used genetic determinants of hyperthyroidism risk, but rather genetic determinants of thyroid stimulating hormone level, which are likely to be different. Moreover, our study described the effect of clinical risk factors on fracture in the general population, and is therefore not generalisable to states or conditions of extreme circumstances known to cause fracture (eg, sustained vitamin D deficiency, rickets, or osteomalacia). Furthermore, our results apply only to 25-hydroxy-vitamin D, and might not necessarily reflect effects of its active form, 1,25-dihydroxy-vitamin D. However, vitamin D supplementation, as is commonly used, acts by influencing 25-hydroxy-vitamin D. Altogether, the course of action for effective fracture prevention relies on establishing vitamin D deficiency and seeking its correction, rather than the widespread use of non-indicated ineffective supplementation.

Strengths and Weaknesses of the Study

To our knowledge, we have generated the largest and most comprehensive assessment of the genetic determinants of fracture risk so far. Moreover, use of the largest GWAS datasets available enabled adequate power to estimate the relation between genetically modified risk factors and fracture. Our study also had limitations. In our mendelian randomisation approach, we were unable to account for the sample overlap between the exposure and outcome GWAS datasets. However, we used powerful instruments to estimate the relation[39] between the risk factors and the outcomes. Therefore, any sample overlap should not significantly bias our findings. Another potential limitation was that the first release of the UK Biobank selected some individuals based on a nested case-control study of smoking and lung function,[70] and is therefore subject to selection bias.[71] But after excluding the UK Biobank from our analyses, we observed no significant differences. Furthermore, the majority of our cohorts were imputed

to HapMap (instead of more comprehensive reference panels), which could have affected the number of identified loci. However, given our power setting, our focus was mainly on common variants (which are well characterised in the HapMap imputation panel). In addition, analysis of large cohorts (UK Biobank and EPIC-Norfolk) imputed to more recent reference panels did not yield additional genome wide significant loci. Moreover, we could not assess several relevant clinical risk factors. For example, body mass index[72-74] could not be assessed in our mendelian randomisation framework because all GWAS analyses of fracture have been adjusted for body weight, preventing any inference assessment on causality. We also lacked power to estimate the casual relation between smoking and alcohol consumption: two potentially key risk factors for fracture. Similarly, we did not evaluate other risk factors that were not modifiable (such as age, sex, parental fracture history and body height), or those that have not been assessed by GWAS to yield genetic instruments for mendelian randomisation studies (such as falls, which are likely to be an important modifiable risk factor for fracture).[75] Furthermore, factors unlikely to be predominantly genetic in origin (eg, occupation) might still have a role in the pathogenesis of fracture but could not be readily assessed through our mendelian randomisation approach. Nevertheless, proxy phenotypes for such risk factors are increasingly been investigated by GWAS (eg, education for occupation), and can be used as robust instruments in future research. In addition, because information on bone mineral density was not available for all study participants who were investigated for fracture, we could not determine directly the degree to which bone mineral density mediated the effect of genetic determinants on fracture risk. Mendelian randomisation is a helpful method to minimise several biases in observational studies, but the possibility of residual pleiotropy could bias estimates in this study. However, the likelihood of this bias is reduced because the mendelian randomisation-Egger regression test showed no clear directional pleiotropy for most of the factors. Lastly, because most of the study population was of European ancestry, results should not be directly generalised to other ethnicities. Similarly, null results of a mendelian randomisation study could be influenced by canalisation, which is defined as compensatory feedback mechanisms that cannot be taken into account.[76] The possible influence of the risk factors on fracture risk might be specifically linked to their complications or management of the disease, which we also could not take into account in mendelian randomisation. As in most epidemiological studies, mendelian randomisation also assumes a linear relation between the risk factor and the outcome, which might not invariably be the case for all risk factors of fracture. Some risk factors, such as vitamin D and estimated calcium intake, could have non-linear threshold associations, as discussed above. Furthermore, we could not account for the dose-response association (eg, between the lactose variant rs4988235 and dairy intake) within our design, or differences in biological effects across different types

of grouped exposures (that is, fermented vs non-fermented types of dairy products). Finally, the non-significant trend observed for vitamin D towards having increased risk of fracture could be attributed to the selection of healthy people (that is, participants with very low levels of vitamin D and fracture, as well as those who are older, frail, and physically impaired, could have been under-represented in the studies included in the GWAS meta-analyses). Therefore, the vitamin D estimates of the current study cannot be generalised to these groups of older people.

Conclusion

From a study of over 500 000 individuals (about 185 000 fracture cases), we provide evidence that the main genetic determinants of osteoporotic fracture also influence bone mineral density, which was the only clinical risk factor to have shown a major effect on fracture risk among the study population assessed. By contrast, we found that other genetically estimated clinical risk factors for fracture, had either a very modest or no effect on fracture risk in the general population. Notably, genetic predisposition to lower levels of vitamin D and estimated calcium intake from dairy sources were not associated with fracture risk. Our study confirms bone mineral density as a pivotal cause of osteoporotic fracture and postulates that, among all the clinical risk factors we evaluated, interventions aimed at increasing bone mineral density are likely to have the most clinically relevant effect on fracture risk reduction.

Detailed acknowledgments and online resources can be found in the published article online

https://www.bmj.com/content/362/bmj.k3225

REFERENCES

1 Melorose J, Perroy R, Careas S. World population prospects. United Nations, 2015. doi:10.1017/ CBO9781107415324.004.

2 Harvey N, Dennison E, Cooper C. Osteoporosis: impact on health and economics [correction in: Nat Rev Rheumatol 2010;6:184]. Nat Rev Rheumatol2010;6:99-105. doi:10.1038/nrrheum.2009.260

3 CooperC, MeltonLJ. Magnitude and impact of osteoporosis and fractures.Academic Press, 1996.

4 Kanis JA, HansD, Cooper C, et al, Task Force of the FRAX Initiative. Interpretation and use of FRAX in clinical practice. Osteoporos Int2011;22:2395-411. doi:10.1007/s00198-011-1713-z

5 NguyenND, Frost SA, Center JR, Eisman JA, Nguyen TV. Development of a nomogram for individualizing hip fracture risk in men and women. Osteoporos Int 2007;18:1109-17. doi:10.1007/ s00198-007-0362-8

6 Nguyen ND, Eisman JA, Center JR, Nguyen TV. Risk factors for fracture in nonosteoporotic men and women. J Clin Endocrinol Metab2007;92:955-62. doi:10.1210/jc.2006-1476

7 Khosla S, Shane E. A crisis in the treatment of osteoporosis. J Bone Miner Res2016;31:1485-7. doi:10.1002/jbmr.2888

8 8Andrew T, Antioniades L, Scurrah KJ, Macgregor AJ, Spector TD. Risk of wrist fracture in women is heritable and is influenced by genes that are largely independent of those influencing BMD. J Bone Miner Res2005;20:67-74. doi:10.1359/JBMR.041015

9 Michaëlsson K, Melhus H, Ferm H, Ahlbom A, Pedersen NL. Genetic liability to fractures in the elderly. Arch Intern Med2005;165:1825- 30. doi:10.1001/archinte.165.16.1825

10 Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol2003;32:1-22. doi:10.1093/ije/dyg070

11 Estrada K, Styrkarsdottir U, Evangelou E, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet2012;44:491-501. doi:10.1038/ng.2249

12 Day FR, Ruth KS, Thompson DJ, et al, PRACTICAL consortium, kConFab Investigators, AOCS Investigators, Generation Scotland, EPIC-InterAct Consortium, LifeLines Cohort Study. Large-scale genomic analyses link reproductive aging to hypothalamic signaling, breast cancer susceptibility and BRCA1-mediated DNA repair. Nat Genet 2015;47:1294-303. doi:10.1038/ng.3412

13 Okada Y, Wu D, Trynka G, et al, RACI consortium, GARNET consortium. Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature 2014;506:376-81. doi:10.1038/ nature12873

14 Jostins L, Ripke S, Weersma RK, et al, International IBD Genetics Consortium (IIBDGC). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature2012;491:119-24. doi:10.1038/nature11582

15 Bradfield JP, Qu H-Q, Wang K, et al. A genome-wide meta-analysis of six type 1 diabetes cohorts identifies multiple associated loci. PLoS Genet 2011;7:e1002293. doi:10.1371/journal. pgen.1002293

16 Porcu E, Medici M, Pistis G, et al. A meta-analysis of thyroidrelated traits reveals novel loci and gender-specific differences in the regulation of thyroid function. PLoS Genet2013;9:e1003266. doi:10.1371/journal.pgen.1003266
17 van Meurs JBJ, Dhonukshe-Rutten RAM, Pluijm SMF, et al. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med2004;350:2033-41. doi:10.1056/NEJMoa032546
18 Willems SM, Wright DJ, Day FR, et al, GEFOS Any-Type of Fracture Consortium. Large-scale GWAS identifies multiple loci for hand grip strength providing biological insights into muscular fitness. Nat Commun2017;8:16015. doi:10.1038/ncomms16015
19 Perry JRB, Day F, Elks CE, et al, Australian Ovarian Cancer Study, GENICA Network, kConFab, LifeLines Cohort Study, InterAct Consortium, Early Growth Genetics (EGG) Consortium. Parent-oforigin-specific allelic associations among 106 genomic loci for age at menarche. Nature2014;514:92-7. doi:10.1038/nature13545
20 Scott RA, Lagou V, Welch RP, et al, DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium. Large-scale association analyses identify new loci influencing glycemic traits and provide insight into the underlying biological pathways. Nat Genet2012;44:991-1005. doi:10.1038/ng.2385
21 Wessel J, Chu AY, Willems SM, et al, EPIC-InterAct Consortium. Low-frequency and rare exome chip variants associate with fasting glucose and type 2 diabetes susceptibility. Nat Commun2015;6:5897. doi:10.1038/ncomms6897
22 Deloukas P, Kanoni S, Willenborg C, et al, CARDIoGRAMplusC4D Consortium, DIAGRAM Consortium, CARDIOGENICS Consortium, MuTHER Consortium, Wellcome Trust Case Control Consortium. Largescale association analysis identifies new risk loci for coronary artery disease. Nat Genet2013;45:25-33. doi:10.1038/ng.2480
23 Morris AP, Voight BF, Teslovich TM, et al, Wellcome Trust Case Control Consortium, Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) Investigators, Genetic Investigation of ANthropometric Traits (GIANT) Consortium, Asian Genetic Epidemiology Network–Type 2 Diabetes (AGEN-T2D) Consortium, South Asian Type 2 Diabetes (SAT2D) Consortium, DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nat Genet2012;44:981- 90. doi:10.1038/ng.2383
24 Mokry LE, Ross S, Ahmad OS, et al. Vitamin D and risk of multiple sclerosis: a Mendelian randomization study [correction in: PLoS Med 2016;13:e1001981]. PLoS Med2015;12:e1001866. doi:10.1371/ journal.pmed.1001866
25 Wang TJ, Zhang F, Richards JB, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet2010;376:180-8. doi:10.1016/S0140-6736(10)60588-0
26 Ding M, Huang T, Bergholdt HK, Nordestgaard BG, Ellervik C, Qi L, CHARGE Consortium. Dairy consumption, systolic blood pressure, and risk of hypertension: Mendelian randomization study [correction in: BMJ 2017;358:j3550]. BMJ 2017;356:j1000. doi:10.1136/bmj. j1000
27 Zheng HF, Forgetta V, Hsu Y-H, et al, AOGC Consortium, UK10K Consortium. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature2015;526:112-7. doi:10.1038/nature14878
28 Mackey DC, Lui L-Y, Cawthon PM, et al, Study of Osteoporotic Fractures (SOF) and Osteoporotic Fractures in Men Study (MrOS) Research Groups. High-trauma fractures and

low bone mineral density in older women and men. JAMA2007;298:2381-8. doi:10.1001/jama.298.20.2381

29 Sanders KM, Pasco JA, Ugoni AM, et al. The exclusion of high trauma fractures may underestimate the prevalence of bone fragility fractures in the community: the Geelong Osteoporosis Study. J Bone Miner Res1998;13:1337-42. doi:10.1359/jbmr.1998.13.8.1337

30 Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Järvelä I. Identification of a variant associated with adult-type hypolactasia. Nat Genet2002;30:233-7. doi:10.1038/ng826

31 Koek WNH, van Meurs JB, van der Eerden BC, et al. The T-13910C polymorphism in the lactase phlorizin hydrolase gene is associated with differences in serum calcium levels and calcium intake. J Bone Miner Res2010;25:1980-7. doi:10.1002/jbmr.83

32 Frank J, Cichon S, Treutlein J, et al. Genome-wide significant association between alcohol dependence and a variant in the ADH gene cluster. Addict Biol 2012;17:171-80. doi:10.1111/j.1369- 1600.2011.00395.x

33 Gelernter J, Kranzler HR, Sherva R, et al. Genome-wide association study of alcohol dependence:significant findings in African- and European-Americans including novel risk loci. Mol Psychiatry2014;19:41-9. doi:10.1038/mp.2013.145

34 Taylor AE, Fluharty ME, Bjørngaard JH, et al. Investigating the possible causal association of smoking with depression and anxiety using Mendelian randomisation meta-analysis: the CARTA consortium. BMJ Open2014;4:e006141. doi:10.1136/ bmjopen-2014-006141 on 3 March 2020 at Erasmus Medical / X51 4300.7802.430. Protected by copyright. http://www.bmj.com/ BMJ: first published as 10.1136/bmj.k3225 on 29 August 2018. Downloaded from RESEARCH the bmj | BMJ 2018;362:k3225 | doi: 10.1136/bmj.k3225 13

35 O'Seaghdha CM, Wu H, Yang Q, et al, SUNLIGHT Consortium, GEFOS Consortium. Meta-analysis of genome-wide association studies identifies six new Loci for serum calcium concentrations. PLoS Genet2013;9:e1003796. doi:10.1371/ journal.pgen.1003796

36 Locke AE, Kahali B, Berndt SI, et al, LifeLines Cohort Study, ADIPOGen Consortium, AGEN-BMI Working Group, CARDIOGRAMplusC4D Consortium, CKDGen Consortium, GLGC, ICBP, MAGIC Investigators, MuTHER Consortium, MIGen Consortium, PAGE Consortium, ReproGen Consortium, GENIE Consortium, International Endogene Consortium. Genetic studies of body mass index yield new insights for obesity biology. Nature 2015;518:197-206. doi:10.1038/nature14177

37 Bulik-Sullivan BK, Loh P-R, Finucane HK, et al, Schizophrenia Working Group of the Psychiatric Genomics Consortium. LD Score regression distinguishes confounding from polygenicity in genomewide association studies. Nat Genet2015;47:291-5. doi:10.1038/ng.3211

38 Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. Genet Epidemiol2013;37:658-65. doi:10.1002/gepi.21758

39 Pierce BL, Burgess S. Efficient design for Mendelian randomization studies: subsample and 2-sample instrumental variable estimators. Am J Epidemiol 2013;178:1177-84. doi:10.1093/aje/kwt084

40 Mokry LE, Ross S, Ahmad OS, et al. Vitamin D and risk of multiple sclerosis: a Mendelian randomization study [correction in: PLoS Med 2016;13:e1001981]. PLoS Med2015;12:e1001866. doi:10.1371/ journal.pmed.1001866

41 Dastani Z, Hivert M-F, Timpson N, et al, DIAGRAM+ Consortium, MAGIC Consortium, GLGC Investigators, MuTHER Consortium, DIAGRAM Consortium, GIANT Consortium, Global B Pgen Consortium, Procardis Consortium, MAGIC investigators, GLGC Consortium. Novel loci for adiponectin levels and their influence on type 2 diabetes and metabolic traits: a multi-ethnic meta-analysis of 45,891 individuals. PLoS Genet 2012;8:e1002607. doi:10.1371/journal. pgen.1002607

42 Johnson T. Efficient calculation for multi-SNP genetic risk scores. 2012; 2012.

43 Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol2015;44:512-25. doi:10.1093/ije/ dyv080

44 Gage SH, Jones HJ, Burgess S, et al. Assessing causality in associations between cannabis use and schizophrenia risk: a twosample Mendelian randomization study. Psychol Med2017;47:971- 80. doi:10.1017/S0033291716003172

45 Brion M-JA, Shakhbazov K, Visscher PM. Calculating statistical power in Mendelian randomization studies. Int J Epidemiol2013;42:1497-501. doi:10.1093/ije/dyt179

46 Richards JB, Rivadeneira F, Inouye M, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 2008;371:1505-12. doi:10.1016/S0140- 6736(08)60599-1

47 Rivadeneira F, Styrkársdottir U, Estrada K, et al, Genetic Factors forOsteoporosis (GEFOS) Consortium. Twenty bone-mineraldensity loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet2009;41:1199-206. doi:10.1038/ ng.446

48 Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, et al. New sequence variants associated with bone mineral density. Nat Genet2009;41:15-7. doi:10.1038/ng.284

49 Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med2008;358:2355-65. doi:10.1056/NEJMoa0801197

50 Duncan EL, Danoy P, Kemp JP, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet2011;7:e1001372. doi:10.1371/journal.pgen.1001372

51 Zheng H-F, Tobias JH, Duncan E, et al. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet 2012;8:e1002745. doi:10.1371/journal.pgen.1002745

52 Medina-Gomez C, Kemp JP, Estrada K, et al. Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet2012;8:e1002718. doi:10.1371/journal. pgen.1002718

53 Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. Am J Hum Genet2012;90:7-24. doi:10.1016/j. ajhg.2011.11.029

54 Ahmad OS, Leong A, Miller JA, et al. A Mendelian randomization study of the effect of type-2 diabetes and glycemic traits on bone mineral density. J Bone Miner Res2017;32:1072-81. doi:10.1002/ jbmr.3063.

55 Richards JB, Zheng H-F, Spector TD. Genetics of osteoporosis from genome-wide association studies: advances and challenges [correction in: Nat Rev Genet 2012;13:672]. Nat Rev Genet2012;13:576-88. doi:10.1038/nrg3228

56 Hurle MR, Nelson MR, Agarwal P, Cardon LR. Trial watch: Impactofgenetically supported target selection on R&D productivity.Nat Rev Drug Discov2016;15:596-7. doi:10.1038/nrd.2016.164

57 Papaioannou A, Morin S, Cheung AM, et al, Scientific Advisory Council of Osteoporosis Canada. 2010 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada: summary. CMAJ2010;182:1864-73. doi:10.1503/cmaj.100771

58 Kanis JA, McCloskey EV, Johansson H, Cooper C, Rizzoli R, Reginster JY, Scientific Advisory Board of the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) and the Committee of Scientific Advisors of the International Osteoporosis Foundation (IOF). European guidance for the diagnosis and management of osteoporosis in postmenopausal women. Osteoporos Int 2013;24:23-57. doi:10.1007/ s00198-012- 2074-y

59 Rooney MR, Harnack L, Michos ED, Ogilvie RP, Sempos CT, Lutsey PL. Trends in use of high-dose vitamin D supplements exceeding 1000 or 4000 international units daily, 1999-2014. JAMA2017;317:2448-50. doi:10.1001/ jama.2017.4392

60 Leong A, Rehman W, Dastani Z, et al, METASTROKE. The causal effect of vitamin D binding protein (DBP) levels on calcemic and cardiometabolic diseases: a Mendelian randomization study. PLoS Med2014;11:e1001751. doi:10.1371/journal. pmed.1001751

61 Li S-S, Gao L-H, Zhang X-Y, et al. Genetically low vitamin D levels, bone mineral density, and bone metabolism markers: a Mendelian randomisation study. Sci Rep2016;6:33202. doi:10.1038/ srep33202

62 Larsson SC, Melhus H, Michaëlsson K. Circulating serum 25-hydroxyvitamin D levels and bone mineral density: Mendelian randomization study. J Bone Miner Res2018;33:840-4. doi:10.1002/jbmr.3389.

63 Larsson SC, Burgess S, Michaëlsson K. Association of genetic variants related to serum calcium levels with coronary artery disease and myocardial infarction. JAMA2017;318:371-80. doi:10.1001/ jama.2017.8981

64 Bauer DC. Clinical practice. Calcium supplements and fracture prevention. N Engl J Med2013;369:1537-43. doi:10.1056/ NEJMcp1210380

65 Bergman GJD, Fan T, McFetridge JT, Sen SS. Efficacy of vitamin D3 supplementation in preventing fractures in elderly women: a meta-analysis. Curr Med Res Opin2010;26:1193-201. doi:10.1185/03007991003659814

66 Bischoff-Ferrari HA, Willett WC, Orav EJ, et al. A pooled analysis of vitamin D dose requirements for fracture prevention [correction in: N Engl J Med 2012;367:481]. N Engl J Med2012;367:40-9. doi:10.1056/NEJMoa1109617

67 Tang BM, Eslick GD, Nowson C, Smith C, Bensoussan A. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis. Lancet2007;370:657-66. doi:10.1016/S0140-6736(07)61342-7

68 Zhao J-G, Zeng X-T, Wang J, Liu L. Association between calcium or vitamin D supplementation and fracture incidence in community-dwelling older adults: a systematic review and meta-analysis. JAMA2017;318:2466-82. doi:10.1001/ jama.2017.19344

69 Schuit SCE, van der Klift M, Weel AEAM, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone2004;34:195-202. doi:10.1016/j. bone.2003.10.001

70 Wain LV, Shrine N, Miller S, et al, UK Brain Expression Consortium (UKBEC), OxGSK Consortium. Novel insights into the genetics of smoking behaviour, lung function, and chronic obstructive pulmonary disease (UK BiLEVE): a genetic association study in UK Biobank. Lancet Respir Med2015;3:769-81. doi:10.1016/S2213- 2600(15)00283-0

71 Munafò MR, Tilling K, Taylor AE, Evans DM, Davey Smith G. Collider scope: when selection bias can substantially influence observed associations. Int J Epidemiol2018;47:226-35. doi:10.1093/ije/ dyx206

72 De Laet C, Kanis JA, Odén A, et al. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporos Int2005;16:1330-8. doi:10.1007/s00198-005-1863-y

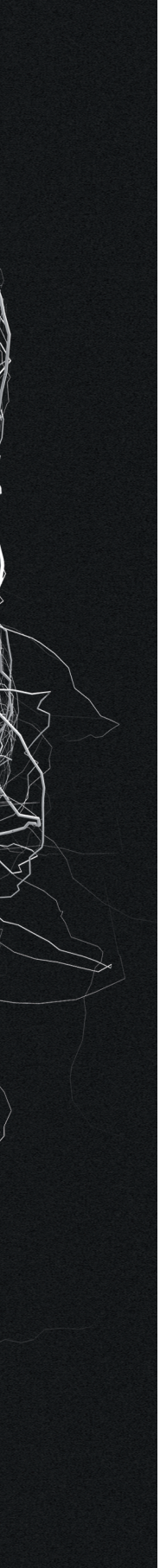
73 Johansson H, Kanis JA, Odén A, et al. A meta-analysis of the association of fracture risk and body mass index in women. J Bone Miner Res2014;29:223-33. doi:10.1002/jbmr.2017

74 Compston JE, Flahive J, Hosmer DW, et al, GLOW Investigators. Relationship of weight, height, and body mass index with fracture on 3 March 2020 at Erasmus Medical / X51 4300.7802.430. Protected by copyright. http://www.bmj.com/ BMJ: first published as 10.1136/bmj.k3225 on 29 August 2018. Downloaded from RESEARCH No commercial re-use: See rights and reprints http://www.bmj.com/permissions Subscribe: http://www.bmj.com/subscribe risk at different sites in postmenopausal women: the Global Longitudinal study of Osteoporosis in Women (GLOW). J Bone Miner Res2014;29:487-93. doi:10.1002/ jbmr.2051

75 El-Khoury F, Cassou B, Charles M-A, Dargent-Molina P. The effectof fall prevention exercise programmes on fall induced injuriesincommunity dwelling older adults: systematic review andmeta-analysis of randomised controlled trials. BMJ2013;347:f6234.

76 Lawlor DA, Harbord RM, Sterne JAC, Timpson N, Davey Smith G. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med2008;27:1133-63. doi:10.1002/sim.3034





2.2.5

Using Mendelian Randomization to Decipher Mechanisms of Bone Disease

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ABSTRACT

Purpose of Review: This review summarizes the basic principles of Mendelian randomization (MR) and provides evidence for the causal effect of multiple modifiable factors on bone outcomes.

Recent Findings: Several studies using MR approach have provided support for the causal effect of obesity on bone mineral density (BMD). Strikingly, studies have failed to prove a causal association between elevated 25(OH) D concentrations and higher BMD in community-dwelling individuals.

Summary: The MR approach has been successfully used to evaluate multiple factors related to bone mineral density variation and/or fracture risk. The MR approach avoids some of the classical observational study limitations and provides more robust causal evidence, ensuring bigger success of the clinical trials. The selection of interventions based on genetic evidence could have a substantial impact on clinical practice.

INTRODUCTION

A fundamental goal of epidemiological (observational) studies is to determine causal factors of diseases. However, in observational studies, we test for association, which by itself does not imply causation. Two logical fallacies cum hoc ergo propter hoc ("with this, therefore because of this") and post hoc ergo propter hoc ("after this, therefore because of this") challenge the interpretation of observational studies. The relationship between exposure (expected cause) and disease (expected outcome) can be distorted by (a) the presence of unmeasured or unaccounted confounders, (b) reverse causation, and a variety of other potential biases. Although proper study designs and analytical approaches can minimize the effect of the aforementioned factors, we still fail to account for most of them. Therefore, interventions based exclusively on evidence derived from association studies might turn out fruitless. Some factors and biomarkers identified in observational studies have failed to be confirmed by large, robust randomized control trials (RCTs). For instance, in the past several years, observational studies[1-3] and one small RCT[4] have provided encouraging evidence for the beneficial effect of the vitamin D and calcium supplements on bone health. Thus, vitamin D and calcium supplementation have been included in the clinical guidelines for osteoporosis management and fracture prevention.[5,6] However, in the past years, from a total of 38 RCTs (14 large and 24 small), the majority failed to detect a benefit of vitamin D and calcium supplements.[7-9] Moreover, a small proportion of the trials have found modest protective effects,[10] and some have even shown an increased harm (e.g., falls, fractures).[11] Even though experimental [randomized] studies are considered to be the gold standard for estimating causality in research,[12] they have their own caveats like limitations due to ethical and technical issues and the exposure cannot be randomized, or being time-consuming and frequently costly.[13] Moreover, the lack of external validity (generalizability of the treatment/condition outcomes) affects the reliability of the results from the RCTs, which may result in flawed policy recommendations. In order to overcome the limitations from the observational and experimental studies, up till now, many methods (conditioning, mechanism-based, natural experiments) for causal inference have been developed that can be easily applied in epidemiological settings and can improve the identification of clinically relevant risk factors. Mendelian randomization is one of them. The aim of this review is to explain the basic principles of Mendelian randomization and provide examples of how Mendelian randomization has been applied to bone research.

CASUAL INFERENCE: MENDELIAN RANDOMIZATION ANALYSIS AND PRINCIPLES

Mendelian randomization plays an important role in causal inference. During conception, parental gametes combine to form a zygote. Each gamete contains a different set of DNA as a result of recombination and independent assortment during conception resulting in genetically defined subgroups of individuals. The Mendelian randomization (MR) design is considered to be analogous to a RCT[14] where instead of random allocation of participants to interventions (treatments or preventive measures) individuals are randomized by nature according to carriership of gene variants that regulate susceptibility to a specific exposure (**Figure 1**). Within both approaches, individuals are divided into random groups balanced across confounding factor(s). Following this principle, genetic variants associated with specific risk factors can be used as a non-confounded proxy to investigate the causal association between the risk factor in question and disease outcomes. Moreover, genetic variants have the advantages of being largely fixed since conception and remain stable throughout life. The expansion of genome wide association studies (GWAS) and improvement in array and imputations panels has enabled well-powered settings facilitating the identification of numerous genetic variants associated with different diseases and complex traits. Such large yield in genetic discoveries propelled by large-scale GWAS has improved considerably the extent of explained trait variance and the prospect of risk prediction of common diseases.[15] This also means that the MR approach leveraged by the abundance of genetic discoveries can now be easily implemented across numerous observational settings. This way, the MR approach can provide prior knowledge before launching RCTs or it can give way to more valid estimates of causal relationships in situations where an RCT cannot be conducted (e.g., smoking and alcohol intake). If MR findings provide evidence of causality for a specific marker, the next step will be to identify the correct biological pathway before performing RCTs. In addition, the MR is a simple and cost-effective method to assess causal relationships between risk factors and health outcomes. In order to obtain unbiased estimates, three key assumptions of MR need to be fulfilled with regard to the instrument: (1) genetic variants are associated with the risk factors or biomarkers under study; (2) genetic variants should not be associated with known confounding factors; and (3) it affects the outcome only through the risk factor and not via other biological pathways (pleiotropy) (**Figure 2**). The first assumption can be easily verified by exploring the data. Even if this assumption holds, we need to test the strength of the association between the genetic variants and exposure (e.g., using partial F statistic) to avoid week instrument bias.[16] The second and third assumption can be difficult to address. First, we cannot estimate the association between the genetic variant and unobserved confounders

(second assumption). However, we can still test the association with observed potential confounding factors or/and search the literature for any reported associations. Second, the presence of pleiotropy (third assumption) can be indirectly detected and corrected by using robust statistical methods [17] (e.g., MR-Egger regression, median weighted). In short, Egger regression assumes that the pleiotropic effect of the variant is independent of the phenotypic effect. If the pleiotropic effects act via a confounder of the "exposure-outcome" association, this assumption will be violated. Moreover, this will affect its associations with both the exposure and the outcome indicating the potential presence of directional pleiotropy. Finally, if the above assumptions hold, the MR can give reliable evidence for causation overcoming the typical pitfalls present in observational studies.

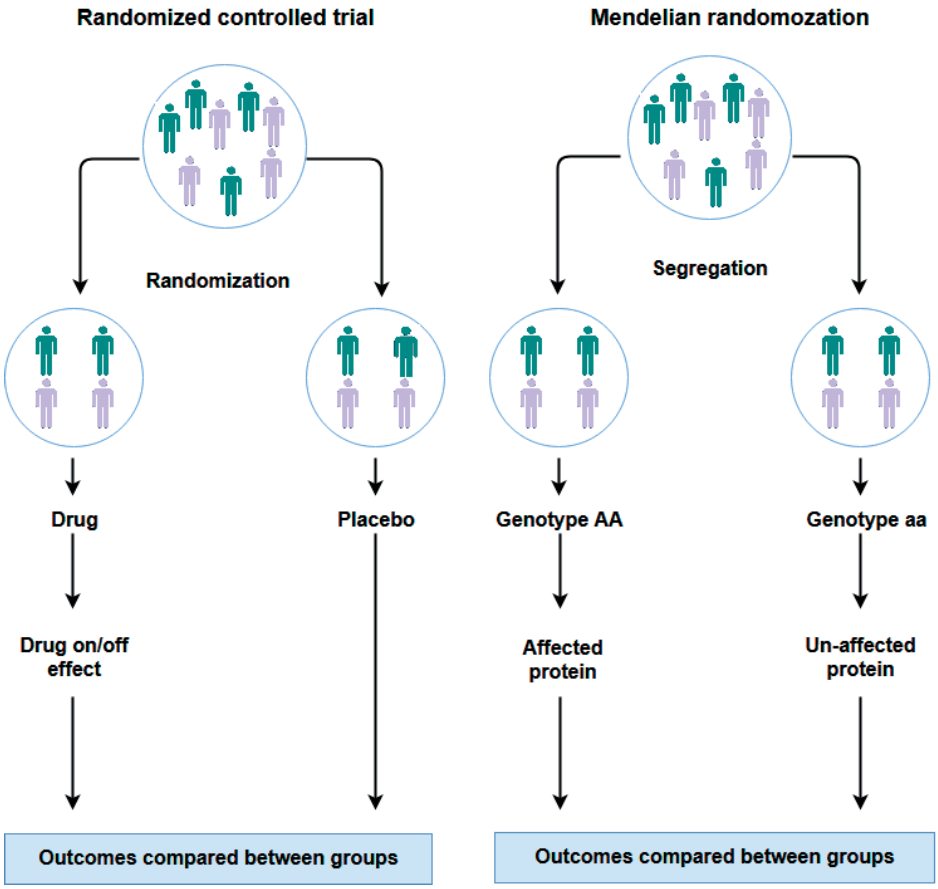


Figure 1 | Comparison of the design of Mendelian randomization study and a randomized controlled trial

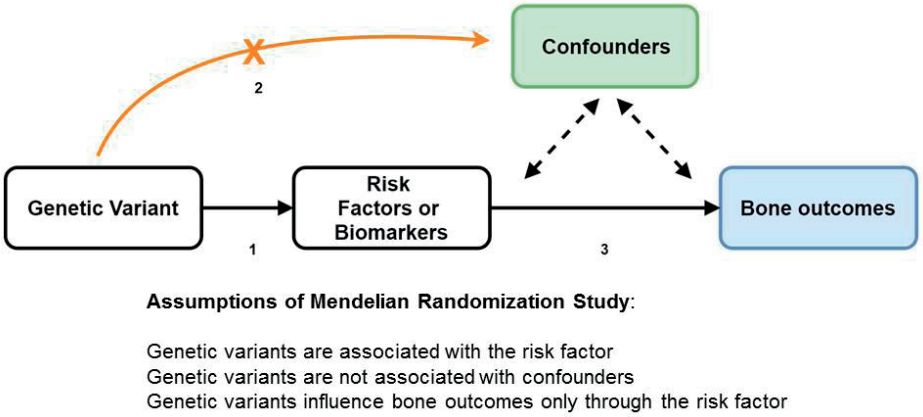


Figure 2 | Directed acyclic graph (DAG) represents the relationship in a typical Mendelian randomization model

MENDELIAN RANDOMIZATION DEBUNKS THE FINDINGS FROM OBSERVATIONAL STUDIES: AN EXAMPLE

It is well established that heavy alcohol drinking during pregnancy has a serious effect on diverse health outcomes of the children. [18] Currently, there is no known safe level of alcohol that can be consumed at any time during pregnancy. However, many women do drink alcohol during pregnancy, generally at a moderate level, as a result of the conflicting messages from the health guidelines. Some of them promote complete abstinence while others recommend moderate drinking. These contradicting messages largely reflect the inconsistent findings from observational studies. For example, some observational studies have found that moderate drinking during pregnancy is even associated with a better cognitive function in children.[19] Nonetheless, the association can be confounded by many socio-economic factors. Taking all these confounders into consideration does attenuate the association, but does not eliminate the effect fully possibly due to residual confounding.[20] Alcohol is metabolized in the body by several alcohol dehydrogenase (ADH) enzymes. Variation in the genes that encode these enzymes influences the metabolic rate of alcohol.[21] Slow metabolizers will be exposed to higher alcohol levels for a longer time compared to fast metabolizers. Thus, it is hypothesized that alleles which increase the metabolism of ethanol will protect against abnormal brain development in infants [20] (as a result of less pronounced exposure to alcohol). For example, researchers of the Avon Longitudinal Study of Parents and Children (ALSPAC, UK-based children cohort) found that four genetic variants in alcohol metabolizing genes were related to low IQ at age 8 in children (carriers of the "slow" metabolizing alleles) whose mothers were drinking during pregnancy [22]. Moreover,

in the same study, Zuccolo et al. [20] found the same association observed by previous studies when using the observational approach, i.e., moderate drinking is associated with increased IQ. However, using the MR method, they found that children of mothers genetically predisposed to drink less were better at school than children of mothers genetically predisposed to drink more [20]. This example illustrates the benefits of the MR approach; considering that most of the observational studies found associations in the same direction, the MR studies disproved them. MR becomes quite relevant in those scenarios where the association under study is confounded by multiple factors (alcohol and cognition in this case). Other examples include studies examining the causal role of CRP, [22] lipoprotein (a), [24] and vitamin D levels [25] with different cardiovascular outcomes, or the association of homocysteine levels with diabetes mellitus. [26]

MENDELIAN RANDOMIZATION IN BONE BIOLOGY

The human skeleton is made of a dynamically growing tissue, essential for locomotion, structural support of soft tissues, and protection of organs. In addition, the skeleton exerts metabolic functions providing a mineral reservoir (primarily for calcium, but also for magnesium and phosphorus) and serves as a depository for cytokines and growth factors that upon release can exert local and systemic effects. Bones are constantly reshaped and renewed throughout the lifespan, through the processes of modeling and remodeling, which are under genetic and environmental control. Modeling occurs in growing bones from birth to the mid-20s, when peak bone mass is achieved. With aging, the imbalance in bone remodeling leads to loss of bone mass and deterioration of bone structure, which predispose to osteoporosis and fracture. An individual's peak bone mass ultimately relates to lifetime risk of fracture (i.e., the higher the peak bone mass, the lower the risk). Yet, partitioning the genetic and environmental influences (risk factors) exerting an effect on bone throughout the lifetime is not trivial. The Mendelian randomization (MR) approach provides means to assess the influence of risk factors on osteoporosis outcomes, including fracture.

To date, the MR approach in the bone field has been applied predominantly to assess cause-effect relationships between different risk factors or biomarkers in relation to bone mineral density as outcome (**Table 1**). Among these body composition factors, inflammation markers and vitamin D levels are the most frequently investigated exposures. In particular, MR analyses have clearly reinforced the role of low BMI as an important risk factor for loss of bone mass.[27*,28,29] Similarly, late puberty [30] and type 2 diabetes and associated glycemic traits [31*] have been shown to exert modest causal effects on bone outcomes; in contrast, genetically increased inflammation markers, [32,33] phosphate [34] (very low powered), and higher urate levels [35,36] had no

causal effect on skeletal outcomes including fracture risk. A recent study has found a modest effect of heel BMD on type 2 diabetes and coronary heart disease, opening the door of evaluating deeper the endocrine function of the bone.[37] Notably, studies investigating the causal role of vitamin D and milk calcium intake showed no evidence of association.[38,39,40*,41]

VITAMIN D AND BONE MINERAL DENSITY

Vitamin D is required for normal bone maturation, formation, and mineralization. Low levels of vitamin D result in hypocalcemia, hypophosphatemia, and hyperparathyroidism, which in turn can lead to impaired mineralization, bone loss, and low BMD levels. Severe lack of vitamin D is known to cause rickets (in children) and osteomalacia (in adults).[42] Nevertheless, the influence of vitamin D on the etiology of low bone mass and the predisposition to develop osteoporosis is still unclear due to inconsistent results across clinical studies. These differences can be attributable to aspects of study design (e.g., study power, type of recruited population, or aspects affecting the vitamin D measurement, like season, thresholds, and assays among others).

There are four SNPs found by GWAS to be strongly associated with 25(OH) D levels, mapping back to genes implicated in vitamin D synthesis, transport, or metabolism. These include rs2282679 in *GC* (association with 25(OH) D: $p = 1.9\times10^{-109}$), rs12785878 near *DHCR7* ($p = 2.1\times10^{-27}$), rs10741657 near *CYP2R1* ($p = 3.3\times10^{-20}$), and rs6013897 in *CYP24A1* ($p = 6.0\times10^{-10}$). [43] The vitamin D-binding protein (DBP), a group-specific component of serum alpha globulin, is encoded by the *GC* gene and it serves as the principal protein carrier for vitamin D and its metabolites [44]. On the other hand, the *DHCR7* gene produces cholesterol, a substrate for vitamin D production. Finally, *CYP2R1* (encoding 25(OH) D synthesis) and *CYP24A1* (encoding 1α25 (OH) 2D inactivation) provide the active form of vitamin D.

Three studies have scrutinized if the relationship between vitamin D and BMD is causal. Leong et al. [38] have investigated the causal relationship between vitamin D-binding protein (DBP) levels and BMD using individual level data (N = 2254) from the Canadian Multicentre Osteoporosis Study (CaMos). In line with their observational results, they showed that DBP might not be a critical player in causal pathways potentially linking vitamin D to BMD. The authors also overcame the sample size limitations of the individual level setting by performing an additional analysis using summary data from the well-powered SUNLIGHT and GEFOS consortia where the null results remained consistent. Furthermore, Li et al. [39] using the four aforementioned vitamin D-associated SNPs found no evidence for a causal effect of vitamin D levels on BMD and bone turnover markers in a population of Chinese postmenopausal women (N =

1824). Finally, Larsson et al. [40*] using data from the GEFOS consortium and UK BioBank study have also recently found that vitamin D levels had no effect on DXA-measured BMD (N = 32,965). However, they observed that elevated vitamin D levels could exert a small decrease in estimated BMD derived from heel ultrasound (N = 142,487). Although the genetic variants have modest effects on vitamin D levels and explain small proportion of the trait variance, the aforementioned studies using summary level data were well powered to investigate causal associations. Very recently, we have also shown lack of a causal relationship between vitamin D levels and fractured risk, investigated in 37,857 cases and 227 116 controls. [45]

These results should be interpreted with caution since the MR efforts have examined a linear relationship between vitmin D levels and BMD. Possible threshold-dependent effects (effects present only in a subgroup with low vitamin D levels) are not examined by this approach. Extreme deficits in vitamin D are known to influence bone metabolism and result in disease (i.e., rickets, osteomalacia). In contrast, the MR setting is drawn in the general population, typically including relatively healthy elderly adults, so the findings might not be applicable to very old and frail people where vitamin D deficiency is frequently present. Another aspect relates to gene x environment (GxE) interactions, which can be challenging to consider in casual inference analyses. It has been postulated that vitamin D levels may be subject to GxE interactions.[46, 47] However, these interactions remain difficult to detect (as testing requires very large sample sizes which are not yet available). Until then, detecting the main effect of a genotype will be more reliable than testing for GxE interactions.[48] Either way, once GxE interactions are detected demonstrating that the exposure differs quantitatively between individuals, then the MR should be restricted to the specific subgroups where the environmental exposure is homogeneous.

LIMITATIONS

In order to obtain unbiased estimates of causality, all three crucial assumptions of MR must be fulfilled. However, the verification of the assumptions is difficult, particularly assessing canalization and pleiotropic effects. In general, the results of MR are said to be robust when multiple methods to assess the MR assumptions are applied and the observed effects still stand. Most importantly, the interpretation of MR studies should be made with caution and with substantial knowledge of the underlying biology. There are multiple factors that can bias the estimates of MR studies: (1) Insufficient power-i.e., the probability that the null hypothesis can be rejected in the presence of true association between the biomarker and disease. If the genetic instrument explains a small proportion of the trait variance, a sufficiently powered sample size

will be required to detect effect and sample size calculations should be performed and considered for the interpretation of the findings. (2) Weak instrument biasstrong instruments will force the association to be independent of confounders. With weak instruments, confounders are not equally balanced between genotype groups and the confounders can explain a given difference in phenotype more strongly than the instruments. Therefore, the instrument should be robustly associated with the exposure of interest. Similarly, an instrument may lack sufficient power when the outcome is only affected by large changes in the exposure. This is particularly relevant for complex traits where common genetic variants typically have a small effect. Therefore, the combined use of multiple variants as instruments will be warranted to achieve sufficient power. (3) Pleiotropy-i.e., when a gene or variant is associated with multiple traits. Even in the presence of a causal effect, the effect can still be due to other factors controlled by the genetic effect. Pleiotropy can be vertical (when genetic variants influence other factors downstream of the primary trait) and horizontal (when the genetic variants influence multiple traits separately). This is nicely illustrated in recent work examining the influence of adiposity and BMD,[27**] where an effect on BMD can be mediated by fat mass, lean mass, or both, drawing the need for careful interpretation of the findings. (4) Population stratification-i.e., differences in genetic structure between subpopulations masked in the population under investigation. The genetic association between the instrument and the outcome should not be driven (or attenuated) by population stratification. Other potential ethnic differences between the discovery (exposure) and the target (outcome) settings (i.e., allele frequencies, linkage disequilibrium structure) should be considered in the interpretation of the MR findings as they reduce the strength of genetic instruments. Canalization/developmental compensation due to operation of compensatory processes during development that may resist the phenotypic changes that result from the genetic variants being used as an instrument.

CLINICAL IMPLICATION

The major advantage of the MR approach is that it can help overcome the expensive costs of running an RCT, by helping in the prioritization of interventions directed towards causal pathways. The selection of interventions based on genetic evidence could have a substantial impact on clinical practice with major considerable utility in primary prevention. In cardiovascular epidemiology, for example, PCSK9 (protein which influences LDL-C levels) has been identified as a potential drug target using MR methods.[49] Recently, phase II clinical trials have proven the safety and efficacy of the monoclonal PCSK9 antibodies.[50] Furthermore, the strengthening of the causal

relationship between modifiable exposures and a wide range of outcomes related to complex diseases can help us improve the drug target identification and validation processes, i.e., the MR approach will contribute to robust determination of the role of factors within biological pathways. For example, a recent study has illustrated how drug mechanisms with genetic support are shown to succeed twice as often as those without it (from phase I to approval).[51] In fact, this is the case for osteoporosis drugs as the highest degree of genetic support for drug target indications was related to the musculoskeletal (BMD), metabolic, and blood categories.[52] In addition, MR can help in identifying adverse effects and drug repurposing.[53] For example, it has been widely recognized that statins, commonly used for prevention of CHD, increase the risk of type 2 diabetes.[54] After the clinical trials, using MR approach, it has been shown that the risk of type 2 diabetes can be partially explained by inhibition of the HMGCR gene (produces enzyme targeted by statins).[55] In principle, this example illustrates (in retrospective) the potential of the MR approach to inform RCT before their execution. Looking back to bone-related phenotypes, most MR methods have evaluated the causality of specific exposures. To date, there are no studies that have investigated the causality of specific drug targets for osteoporosis. One novel osteoporotic treatment is the use of Romosozumab, a monoclonal antibody that targets sclerostin. However, recent trials have shown that Romosozumab is associated with (small yet real) increased risk of cardiovascular adverse events. This way, MR studies are warranted to evaluate the causal relation of Romosozumab treatment with this adverse effect, by investigating whether variations in the SOST gene are associated with cardiovascular or other adverse events.

CONCLUSIONS

The Mendelian randomization (MR) approach is a robust strategy to determine causal relationships between risk factors and diverse health-related outcomes, including bone health. While still in its infancy, the MR approach has been used to evaluate multiple factors mostly related to bone mineral density variation and a few for fracture risk. Given the advent of large-scale GWAS identifying hundreds to thousands of genetic variants robustly associated with bone traits, together with the clear benefits of the MR approach to prioritize interventions of RCT, repurpose existing medications, and prediction of adverse effects, it is expected that many of the unsolved epidemiological questions of observational studies will be solved and better treatments for patients will emerge in the clinic.

REFERENCES

Papers of particular interest, published recently, have been highlighted as: * Of importance ** Of major importance

1. Ooms ME, Roos JC, Bezemer PD, van der Vijgh WJ, Bouter LM, Lips P. Prevention of bone loss by vitamin D supplementation in elderly women: a randomized double-blind trial. J Clin Endocrinol Metab [Internet] 1995;80(4):1052–1058.
2. Dawson-Hughes B, Harris SS, Krall EA, Dallal GE, Falconer G, Green CL. Rates of bone loss in postmenopausal women randomly assigned to one of two dosages of vitamin D. Am J Clin Nutr [Internet] 1995;61(5):1140–1145. doi: 10.1093/ajcn/61.5.1140.
3. Aloia JF, Vaswani A, Yeh JK, Ross PL, Flaster E, Dilmanian FA. Calcium supplementation with and without hormone replacement therapy to prevent postmenopausal bone loss. Ann Intern Med [Internet] 1994;120(2):97–103. doi: 10.7326/0003-4819-120-2-199401150-00001.
4. Dawson-Hughes B, Harris SS, Krall EA, Dallal GE. Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. N Engl J Med [Internet] 1997;337(10):670–676. doi: 10.1056/NEJM199709043371003.
5. Papaioannou A, Morin S, Cheung AM, Atkinson S, Brown JP, Feldman S, et al. 2010 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada: summary. Can Med Assoc J [Internet] 2010;182(17):1864–1873. doi: 10.1503/cmaj.100771.
6. Camacho PM, Petak SM, Binkley N, Clarke BL, Harris ST, Hurley DL, et al. American Association of Clinical Endocrinologists and American College of Endocrinology Clinical Practice guidelines for the diagnosis and treatment of postmenopausal osteoporosis — 2016. Endocr Pract [Internet] 2016;22(Supplement 4):1–42. doi: 10.4158/EP161435. GL. Lips P, Graafmans WC, Ooms ME, Bezemer PD, Bouter LM. Vitamin D supplementation and fracture incidence in elderly persons. A randomized, placebo-controlled clinical trial. Ann Intern Med [Internet] 1996;124(4):400–406. doi: 10.7326/0003-4819-124-4-199602150-00003. [PubMed] [CrossRef] [Google Scholar]
7. Khaw K-T, Stewart AW, Waayer D, Lawes CMM, Toop L, Camargo CA, et al. Effect of monthly high-dose vitamin D supplementation on falls and non-vertebral feractures: secondary and post-hoc outcomes from the randomised, double-blind, placebo-controlled ViDA trial. Lancet Diabetes Endocrinol [Internet] 2017;5(6):438–447. doi: 10.1016/S2213-8587(17)30103-1.
8. Grant AM, Avenell A, Campbell MK, McDonald AM, MacLennan GS, McPherson GC, et al. Oral vitamin D3 and calcium for secondary prevention of low-trauma fractures in elderly people (Randomised Evaluation of Calcium Or vitamin D, RECORD): a randomised placebo-controlled trial. Lancet [Internet]. 2005;365(9471):1621–1628. doi: 10.1016/S0140-6736(05)63013-9.
9. Jackson RD, LaCroix AZ, Gass M, Wallace RB, Robbins J, Lewis CE, et al. Calcium plus vitamin D supplementation and the risk of fractures. N Engl J Med [Internet] 2006;354(7):669–683. doi: 10.1056/NEJMoa055218.
10. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women. JAMA [Internet] 2010;303(18):1815. doi: 10.1001/jama.2010.594. Jones DS, Podolsky SH. The history and

fate of the gold standard. Lancet [Internet]. 2015;385(9977):1502–1503. doi: 10.1016/S0140-6736(15)60742-5.

11. Evans DM, Davey Smith G. Mendelian randomization: new applications in the coming age of hypothesis-free causality. Annu Rev Genomics Hum Genet [Internet] 2015;16(1):327–350. doi: 10.1146/annurev-genom-090314-050016. [PubMed] [CrossRef] [Google Scholar]
12. Nitsch D, Molokhia M, Smeeth L, DeStavola BL, Whittaker JC, Leon DA. Limits to causal inference based on Mendelian randomization: a comparison with randomized controlled trials. Am J Epidemiol [Internet] 2006;163(5):397–403. doi: 10.1093/aje/kwj062.
13. Visscher PM, Wray NR, Zhang Q, Sklar P, McCarthy MI, Brown MA, et al. 10 years of GWAS discovery: biology, function, and translation. Am J Hum Genet [Internet] 2017;101(1):5–22. doi: 10.1016/j.ajhg.2017.06.005. Davies NM, Smith GD, Windmeijer F, Martin RM. Issues in the reporting and conduct of instrumental variable studies. Epidemiology [Internet] 2013;24(3):363–369. doi: 10.1097/EDE.0b013e31828abafb.
14. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol. 2015;44(June):512–525. doi: 10.1093/ije/dyv080. Jones KL, Smith DW, Ulleland CN, Streissguth P. Pattern of malformation in offspring of chronic alcoholic mothers. Lancet (London, England) [Internet] 1973;1(7815):1267–1271. doi: 10.1016/S0140-6736(73)91291-9.
15. Kelly Y, Iacovou M, Quigley M, Gray R, Wolke D, Kelly J, et al. Light drinking versus abstinence in pregnancy - behavioural and cognitive outcomes in 7-year-old children: a longitudinal cohort study. BJOG An Int J Obstet Gynaecol [Internet] 2013;120(11):1340–1347. doi: 10.1111/1471-0528.12246.
16. Zuccolo L, Lewis SJ, Davey Smith G, Sayal K, Draper ES, Fraser R, et al. Prenatal alcohol exposure and offspring cognition and school performance. A 'Mendelian randomization' natural experiment. Int J Epidemiol [Internet] 2013;42(5):1358–1370. doi: 10.1093/ije/dyt172.
17. Birley Andrew J., James Michael R., Dickson Peter A., Montgomery Grant W., Heath Andrew C., Martin Nicholas G., Whitfield John B. ADH single nucleotide polymorphism associations with alcohol metabolism in vivo. Human Molecular Genetics. 2009;18(8):1533–1542. doi: 10.1093/hmg/ddp060. Lewis SJ, Zuccolo L, Davey Smith G, Macleod J, Rodriguez S, Draper ES, et al. Fetal alcohol exposure and IQ at age 8: evidence from a population-based birth-cohort study. PLoS One [Internet] 2012;7(11):e49407. doi: 10.1371/journal.pone.0049407.
18. C Reactive Protein Coronary Heart Disease Genetics Collaboration (CCGC) CRPCHDGC. Wensley F, Gao P, Burgess S, Kaptoge S, Di Angelantonio E, et al. Association between C reactive protein and coronary heart disease: Mendelian randomisation analysis based on individual participant data. BMJ [Internet] 2011;342:d548. doi: 10.1136/bmj.d548.
19. Kamstrup PR, Tybjaerg-Hansen A, Steffensen R, Nordestgaard BG. Genetically Elevated Lipoprotein (a) and increased risk of myocardial infarction. JAMA [Internet] 2009;301(22):2331. doi: 10.1001/jama.2009.801.
20. Manousaki D, Mokry LE, Ross S, Goltzman D, Richards JB. Mendelian randomization studies do not support a role for vitamin D in coronary artery disease. Circ Cardiovasc Genet [Internet] 2016;9(4):349–356.

21. Kumar J, Ingelsson E, Lind L, Fall T. No evidence of a causal relationship between plasma homocysteine and type 2 diabetes: a mendelian randomization study. Front Cardiovasc Med [Internet] 2015;2:11. Kemp JP, Sayers A, Smith GD, Tobias JH, Evans DM. Using Mendelian randomization to investigate a possible causal relationship between adiposity and increased bone mineral density at different skeletal sites in children. Int J Epidemiol [Internet] 2016;45(5):1560–1572. doi: 10.1093/ije/dyw079. [PMC free article]
22. Timpson NJ, Sayers A, Davey-Smith G, Tobias JH. How does body fat influence bone mass in childhood? A Mendelian randomization approach. J Bone Miner Res [Internet] 2009;24(3):522–533. doi: 10.1359/jbmr.081109. Warodomwichit D, Sritara C, Thakkinstian A, Chailurkit L-O, Yamwong S, Ratanachaiwong W, et al. Causal inference of the effect of adiposity on bone mineral density in adults. Clin Endocrinol (Oxf) [Internet] 2013;78(5):694–699. doi: 10.1111/cen.12061. [PubMed] [CrossRef] [Google Scholar]
23. Cousminer DL, Mitchell JA, Chesi A, Roy SM, Kalkwarf HJ, Lappe JM, et al. Genetically determined later puberty impacts lowered bone mineral density in childhood and adulthood. J Bone Miner Res [Internet]. 2017; Available from: http://doi.wiley.com/10.1002/jbmr.3320
24. Ahmad OS, Leong A, Miller JA, Morris JA, Forgetta V, Mujammami M, et al. A Mendelian randomization study of the effect of type-2 diabetes and glycemic traits on bone mineral density. J Bone Miner Res [Internet] 2017;32(5):1072–1081. doi: 10.1002/jbmr.3063.
25. Oei L, Campos-Obando N, Dehghan A, Oei EHG, Stolk L, van Meurs JBJ, et al. Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study. Osteoporos Int [Internet] 2014;25(4):1247–1254. doi: 10.1007/s00198-013-2578-0.
26. Huang JV, Schooling CM. Inflammation and bone mineral density: a Mendelian randomization study. Sci Rep [Internet] 2017;7(1):8666. doi: 10.1038/s41598-017-09080-w.
27. Pekkinen M, Laine CM, Mäkitie R, Leinonen E, Lamberg-Allardt C, Viljakainen H, et al. FGF23 gene variation and its association with phosphate homeostasis and bone mineral density in Finnish children and adolescents. Bone [Internet] 2015;71:124–130. doi: 10.1016/j.bone.2014.10.013.
28. Dalbeth N, Topless R, Flynn T, Cadzow M, Bolland MJ, Merriman TR. Mendelian randomization analysis to examine for a causal effect of urate on bone mineral density. J Bone Miner Res [Internet] 2015;30(6):985–991. doi: 10.1002/jbmr.2434.
29. Xiong A, Yao Q, He J, Fu W, Yu J, Zhang Z. No causal effect of serum urate on bone-related outcomes among a population of postmenopausal women and elderly men of Chinese Han ethnicity--a Mendelian randomization study. Osteoporos Int [Internet] 2016;27(3):1031–1039. doi: 10.1007/s00198-015-3341-5.
30. Gan W, Clarke RJ, Mahajan A, Kulohoma B, Kitajima H, Robertson NR, et al. Bone mineral density and risk of type 2 diabetes and coronary heart disease: a Mendelian randomization study. Wellcome Open Res [Internet] 2017;2:68. doi: 10.12688/wellcomeopenres.12288.1.
31. Leong A, Rehman W, Dastani Z, Greenwood C, Timpson N, Langsetmo L, et al. The causal effect of vitamin D binding protein (DBP) levels on calcemic and cardiometabolic diseases: a Mendelian randomization study. Minelli C, editor. PLoS Med [Internet] 2014;11(10):e1001751. doi: 10.1371/journal.pmed.1001751. Li S-S, Gao L-H, Zhang X-Y, He J-W, Fu W-Z, Liu Y-J, et al. Genetically low vitamin D levels, bone mineral density, and bone metabolism markers: a Mendelian randomisation study. Sci Rep [Internet]

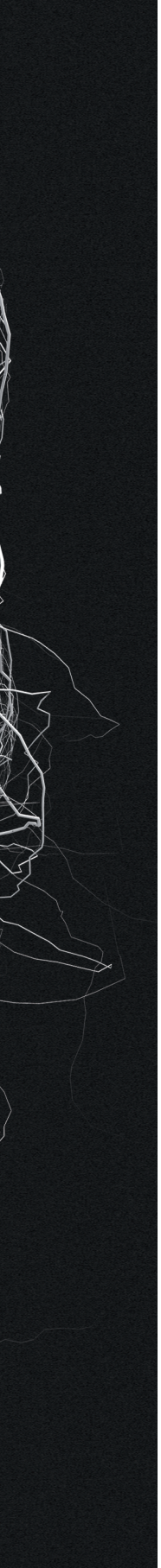
2016;6(1):33202. doi: 10.1038/srep33202. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

32. • Larsson SC, Melhus H, Michaëlsson K. Circulating serum 25-hydroxyvitamin D levels and bone mineral density: Mendelian randomization study. J Bone Miner Res [Internet]. 2018; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29338102. Well-powered study that does not support the causal association between genetically elevated 25OHD concentrations and higher BMD in generally healthy populations.
33. Yang Q, Lin SL, Au Yeung SL, Kwok MK, Xu L, Leung GM, et al. Genetically predicted milk consumption and bone health, ischemic heart disease and type 2 diabetes: a Mendelian randomization study. Eur J Clin Nutr [Internet]. 2017
34. Holick MF. Vitamin D deficiency. N Engl J Med [Internet] 2007;357(3):266–281. doi: 10.1056/NEJMra070553. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet [Internet] 2010;376(9736):180–188. doi: 10.1016/S0140-6736(10)60588-0.
35. Haddad JG. Transport of vitamin D metabolites. Clin Orthop Relat Res [Internet] 1979;142:249–261. Trajanoska K, Morris J, et al., Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study. BMJ 2018
36. Graafmans WC, Lips P, Ooms ME, Van Leeuwen JPTM, Pols HAP, Uitterlinden AG. The effect of vitamin D supplementation on the bone mineral density of the femoral neck is associated with vitamin d receptor genotype. J Bone Miner Res [Internet] 1997;12(8):1241–1245. doi: 10.1359/jbmr.1997.12.8.1241. [PubMed] [CrossRef] [Google Scholar]
37. Elnenaei MO, Chandra R, Mangion T, Moniz C. Genomic and metabolomic patterns segregate with responses to calcium and vitamin D supplementation. Br J Nutr [Internet] 2011;105(01):71–79. doi: 10.1017/S0007114510003065.
38. Smith GD, Shah E. Mendelian randomization: genetic variants as instruments for strengthening causal inference in observational studies. In: National Research Council (US) Committee on Advances in Collecting and Utilizing Biological Indicators and Genetic Information in Social Science Surveys, Weinstein M, Vaupel JW, Wachter KW, editors. Biosocial Surveys. Washingt: Natl Acad. 2008.
39. Cohen JC, Boerwinkle E, Mosley TH, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med [Internet] 2006;354(12):1264–1272. doi: 10.1056/NEJMoa054013. Koren MJ, Lundqvist P, Bolognese M, Neutel JM, Monsalvo ML, Yang J, et al. Anti-PCSK9 monotherapy for hypercholesterolemia. J Am Coll Cardiol [Internet] 2014;63(23):2531–2540. doi: 10.1016/j.jacc.2014.03.018.
40. Nelson MR, Tipney H, Painter JL, Shen J, Nicoletti P, Shen Y, et al. The support of human genetic evidence for approved drug indications. Nat Genet [Internet] 2015;47(8):856–860. doi: 10.1038/ng.3314. Hurle MR, Nelson MR, Agarwal P, Cardon LR. Impact of genetically supported target selection on R&D productivity. Nat Rev Drug Discov [Internet]. 2016; Available from: http://www.nature.com/doifindeer/10.1038/nrd.2016.187
41. Walker VM, Davey Smith G, Davies NM, Martin RM. Mendelian randomization: a novel approach for the prediction of adverse drug events and drug repurposing opportunities. Int J Epidemiol [Internet] 2017;46(6):2078–2089. doi: 10.1093/ije/dyx207.
42. Sattar N, Preiss D, Murray HM, Welsh P, Buckley BM, de Craen AJM, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet (Lon-

don, England) [Internet] 2010;375(9716):735–742. doi: 10.1016/S0140-6736(09)61965-6.

43. Swerdlow DI, Preiss D, Kuchenbaecker KB, Holmes MV, Engmann JEL, Shah T, et al. HMG-coenzyme A reductase inhibition, type 2 diabetes, and bodyweight: evidence from genetic analysis and randomised trials. Lancet [Internet] 2015;385(9965):351–361. doi: 10.1016/S0140-6736(14)61183-1.

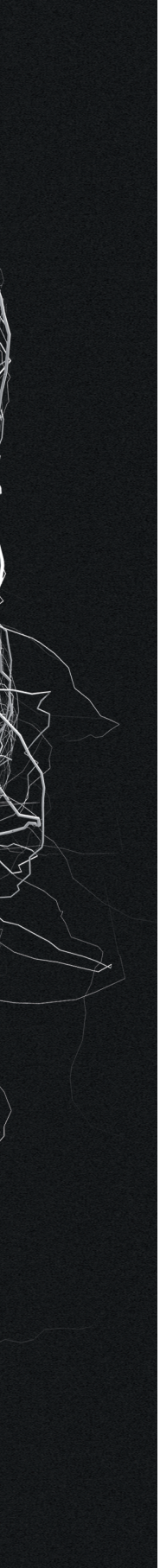




3

Determinants of sarcopenia





3.1

Association between muscle force and power with different pQCT and DXA-derived parameters in middle-aged adults

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In preparation

ABSTRACT

Jumping mechanography (JM) and peripheral Quantitative Computed Tomography (pQCT) have recently gained interest as powerful techniques for the assessment of muscle function and bone geometry. These techniques constitute useful tools for the investigation of the muscle-bone relationship. The aim of this study was to evaluate the association between JM-derived muscle force and power with different pQCT- and DXA-derived parameters. Data was obtained from 568 participants (mean age: 49.8±4.99, 52% female) from a population-based cohort with available data for cross-sectional analysis. Maximum muscle force and power was assessed using single 2-legged jump. pQCT scans were performed at 4% and 66% of the tibial length, measuring bone geometry parameters such as cortical and trabecular bone density, area and circumference. Next, dual-energy X-ray absorptiometry (DXA) was performed and areal bone mineral density (aBMD), lean mass and fat mass were derived. All variables were standardized prior linear regression analyses with standardized β-coefficients presented; corrected for age, sex and weight. Our results showed strong positive association between maximum force and trabecular density at 4% (β: 0.174; 95% CI: 0.033 to 0.315), cortical area (β: 0.192; 95% CI: 0.088 to 0.295) and thickness (β: 0.077; 95% CI:0 .047 to 0.306) at 66%, but not with cortical density at 66% (β: 0.006; 95% CI: -0.127 to 0.139), endosteal circumference (β: -0.022; 95% CI: -0.159 to 0.115) and periosteal circumference (β:0.037; 95% CI: -0.085 to 0.158). Maximum power followed the same trend, and was also associated with periosteal circumference (β: 0.153; 95% CI: 0.023 to 0.282). Finally, muscle force and power were also associated with all DXA-derived bone and body composition parameters. In conclusion, maximum force and power are associated with specific pQCT bone parameters and DXA outcomes. Therefore, they may serve as important clinical markers for musculoskeletal decline in older adults.

INTRODUCTION

Osteoporosis, characterized by loss of bone mineral density (BMD) and deterioration of bone tissue, is a major risk factor for fragility fractures. [1,2] The loss of BMD advances with age, which predisposed towards increased prevalence of osteoporosis and fragility factures in elderly people. Recognition of fracture risk and subsequent fracture prevention among the elderly might be vital for their survival as bone fractures have been associated with increased morbidity and mortality. [3-5] As the elderly population continues to grow, better screening and prevention strategies of osteoporosis and its sequels is needed.

Multiple studies have shown a positive association between muscle mass and bone mass [6-8] as well as between muscle strength and BMD. [9-11] This relationship between bone and muscle can be explained by the mechanostat theory. [12-15] The theory postulates that BMD increases as a result of mechanical load-induced strains, with peak muscle forces thought to be the main cause of strains. This allows for the bone to adapt to the body's own maximum force. For this reason, bone mass and strength increase in individuals with high peak muscle force. [16] On the other hand, low peak muscle force is associated with lower BMD. Muscle dysfunction is highly important in assessing risk of fragility fracture. Muscle weakness not only increases falling risk but also it increases chances of fracture as a result of a fall as well due to lower BMD, thus doubling the risk of future fracture. This acknowledges the importance of the muscle-bone unit as a whole in preventing fragility fractures and presents an opportunity for improvement of screening for fragility. [17]

This theory has led to further investigation of the muscle-bone unit as a possible tool in identifying low BMD individuals. Recent research has used various methods to assess muscle and bone, presenting associations between multiple combinations of muscle and bone parameters. The methods of assessing bone structure are limited to Dual X-ray Absorptiometry (DXA) and peripheral Quantitative Computed Tomography (pQCT). While Dual X-ray Absorptiometry (DXA) has traditionally been the main method in assessing BMD and diagnosing osteoporosis. [18] Where DXA is limited by its two-dimensional nature and primarily produces areal BMD, pQCT scans are three dimensional and more accurate. The pQCT yields volumetric BMD and distinguishes trabecular from cortical parameters. pQCT is a useful tool for research purposes due to its accurate and extensive results and form a good basis for comparing muscle to bone. Although correlations between DXA and pQCT have been found, the techniques seem better suited alongside each other rather than instead of each other. [18] Therefore, the interest in peripheral Quantitative Computed Tomography (pQCT) has increased as it provides more powerful means to assess the bone parameters. [20-22] On the other hand, multiple methods exist to assess muscle functioning such as, questionnaires

to determine current physical activity, pQCT examines muscle cross-sectional area (mCSA), DXA produces lean body mass and various equipment to estimate grip and leg strength and Jumping Mechanography (JM). [23-32] The latter one is used to assess peak jump power/force which, as mentioned before, are assumed to be a main cause of the strains that influence bone makeup. Therefore, a method capable of measuring peak forces is preferred in the assessment of the muscle-bone unit. Additionally, JM is safe and easy and results are easily reproduced. [33-34] This makes JM a promising method in predicting bone mass and strength.

The current method of using DXA's BMD measurements to identify individuals at risk of future fracture are inadequate as most fractures currently occur in individuals who do not have osteoporosis. [35] The combination of pQCT and JM possibly contributes to better predictions of future fracture, but current information about these methods is lacking. Large, population based descriptive data for both methods is missing as well as information about the association between JM and bone parameters, and thus about JM's possible use as a screening too, is limited as well. The aims of this study are therefore 1) to report descriptive information for both pQCT and JM from large population bases cohort and 2) to investigate the association between different pQCT DXA and JM derived parameters.

MATERIAL AND METHODS

Study Population

This present study was embedded within the Rotterdam Study, a prospective population-based cohort that started in 1990[36]. The study investigates risk factors in an ageing population in order to increase recognition of chronic diseases that are frequent among the elderly. Residents of the Ommoord district in Rotterdam in The Netherlands have been recruited for participation since 1990, when the first cohort (n=7,983) started with participants aged 55 years or older. The most recent fourth cohort (n=±4,000) started 2016 and has recruited residents aged between 40 and 55 years. Initially, participants are interviewed at home followed by extensive examination at the study center in the Ommoord district. Follow-up surveys and examination take place in cycles every 3 to 4 years. Examinations focus on imaging and collecting biomarkers, both used for further analysis. The examination procedure is conducted by dedicated research personnel. All data is collected by the research center. Anthropomorphic data are collected at baseline and updated with every round of examinations. These data include age, height, weight and sex. The medical ethics committee of the Erasmus MC (registration number MEC 02.1015) has approved the study design of the Rotterdam Study and written consent is obtained before participation.

Peripheral Quantative Computed Tomography (pQCT)

pQCT has recently been added to the Rotterdam Study's examination protocol. The pQCT scanner (XCT2000 from Stratec Medizintechnik, Pforzheim, Germany) is used to examine bone, muscle and fat characteristics in the lower leg. Scans are performed at distal (4%) and midshaft (66%) of tibia site. Tibial length is measured from inner ankle to the top of the tibia. Scan slices are 3 mm thick. Legs are fixated during scans to prevent movement. Scans are processed by software developed by the manufacturer to produce test results. Trabecular variables are derived from 4% scans, while cortical and cross-sectional area measurements are derived from 66% scans. Density is measured as volume BMD (vBMD). Endosteal and periosteal circumference as well as cortical thickness have been calculated using the average radius of the inner and outer perimeter of the cortical bone. The inner (r) and outer (R) radius was calculated using the formulas:

$$R = \sqrt{((\text{Total bone area})/\pi)}$$

$$r = \sqrt{(((\text{Total bone area} - \text{cortical bone area}))/\pi)}$$

The cortical thickness is the difference between the inner and outer radius. The inner radius was used to calculate the endosteal circumference and the outer radius for the periosteal circumference using the formula:

$$\text{Circumference} = 2 * \text{radius} * \pi$$

To prevent processing errors and inaccurate scans, each scan was visually inspected. Scans with moderate to severe movements were excluded and processing errors were corrected manually where possible. Reported pQCT outcomes are total bone area at 4% (TotA4) and 66% (TotA66), cortical bone area (CrtA), Muscle Cross-Sectional Area (MCSA) and Fat Cross-Sectional Area (FCSA) in mm^2. Endosteal circumference (EndoC), periosteal circumference (PeriC) and Cortical Thickness (CrtTh) are reported in mm. Cortical bone Density (CrtDen) and Trabecular Density (TrbDen) are reported in mg/ccm and Stress-Strain Index (SSI) is reported in mm^3.

Jumping Mechanography (Ground force reaction platform)

A Leonardo Mechanograph Ground Reaction Force Platform (GRFP) was used to test muscle strength through measurements of muscle force and power. The participant is asked to perform a single 2-legged jump (s2LJ), this type of jump is most suitable for determining maximum power output. The participant is instructed to jump as high as possible using both legs and to land upon both forefeet and stand still on both feet for three seconds, completing the single 2-legged jump. Counter movement (knee bending) prior to the jump was allowed to increase jump height. Because of a small

learning curve in performing the jump, the jump is repeated until 3 proper jumps are performed. The highest jump of each participant was used in our analysis. Test outcomes include jump height in cm, maximum force (MaxF) in kilonewtons, maximum power (MaxP) in kilowatts and relative force and power (newtons/watts per kilogram bodyweight).

Dual X-ray Absorptiometry (DXA)

DXA was used to determine areal BMD, lean mass and fat mass. BMD measurements of the femoral neck, the whole femur and the whole body were available. Total lean and fat mass measurements were available as well as lean mass of the arms and legs. DXA outcomes are total lean mass (TLM), total fat mass (FM), arms lean mass (ALM) and legs lean mass (LLM) in kg as well as total BMD (TBMD), spine BMD (SBMD), Femur BMD (FBMD) and Femoral Neck BMD (FNBMD) in g/cm^2.

STATISTICAL METHODS

Before the start of statistical analysis, scatterplots and histograms are checked for distribution and to identify outliers. Outliers are checked for test quality, tests with poor quality are excluded. Descriptives are reported as means with standard deviation for the main outcomes of pQCT, JM and DXA for both male and female in addition to the total population. Partial pearson correlation tests adjusted for age and sex were performed between all major outcomes and correlation coefficients are reported. Multiple linear regression models were used to test for associations between maximum force and power and bone parameters as measured by pQCT and DXA. Model 1 was adjusted for age and sex while in model 2 weight was additionally added. All predictors and outcomes were standardized before regression analysis, thus standardized β-coefficients are presented. Analysis was performed using SPSS (version 24.0.0.1).

RESULTS

Study Population

Data for all three methods was available for 568 participants. The mean age of the study population was 49.48 (SD 4.77) years, 50.8% (n=289) being female. Average weight was 70.79 kg (SD 12.21) and height 167.53 cm (SD 6.57). Study descriptives are presented in Table 1.

Table 1 | Descriptives characteristics of the study population stratified by sex

	Total (n=568)		Male (n=279)		Female (n=289)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Demographics						
Age (years)	49.8	5.0	50.2	5.2	49.5	4.8
Weight (kg)	79.1	15.5	87.8	13.3	70.8	12.6
Height (cm)	174.2	9.6	181.2	7.0	167.5	6.5
BMI (kg/m^2)	25.9	3.9	26.7	3.6	25.2	4.1
Jumping Mechanography						
Jump height (cm)	33.6	8.0	38.4	6.9	29.0	6.0
MaxF (kN)	1.58	0.36	1.79	0.32	1.37	0.27
RelF (N/kg)	19.9	2.7	20.4	2.5	19.4	2.8
MaxP(kW)	2.73	0.87	3.40	0.69	2.09	0.42
RelP (W/kg)	34.2	7.5	38.8	6.4	29.7	5.5
pQCT						
TotArea4 (mm^2)	1,078	172	1,187	142	973	126
TrbDen4 (mg/ccm)	227	40	242	40	213	34
TotArea66 (mm^2)	975	220	1,101	191	854	173
CrtArea66 (mm^2)	292	66	335	55	251	47
CrtDen66 (mg/mm^3)	1,066	40	1,059	35	1,073	44
CrtTh (mm)	2.9	0.7	3.2	0.6	2.7	0.6
EndoC (mm)	92	14	97	12	86	13
PeriC (mm)	110	12	117	10	103	10
MuscA (mm^2)	6,045	1,224	6,844	975	5,273	904
FatA (mm^2)	3,295	1,358	2,565	892	3,999	1,359
SSI (mm^3)	3,305	858	3888	714	2742	558
DXA						
LM (kg)	49.7	10.4	58.4	6.6	41.3	5.1
FM (kg)	25.8	8.6	25.8	8.5	25.9	8.8
ALM (kg)	64.5	2.0	8.2	1.2	4.8	0.8
LLM (kg)	17.5	3.9	20.6	2.7	14.5	2.2
TBMD (g/cm^2)	1.17	0.12	1.22	0.11	1.13	0.11
SBMD (g/cm^2)	1.01	0.14	1.04	0.13	0.97	0.13
FNBMD (g/cm^2)	0.96	0.13	0.99	0.13	0.93	0.13

Correlation

Correlation between different pQCT, JM and DXA parameters are shown in **Figure 1.** Maximum jumping force showed strong correlation with multiple bone parameters, most notably cortical area (0.39, $p < 0.001$), total BMD (0.40, $p < 0.001$) and cortical thickness (0.26, $p < 0.001$).

Maximum jumping power was also most notably correlated with cortical area (0.36, $p < 0.001$), total BMD (0.37, $p < 0.001$) and cortical thickness (0.23, $p < 0.001$). Interestingly, total lean mass correlated better with most bone parameters than maximum force and power **Figure 1**. Force and power correlated strongly with other muscle parameters such as muscle cross-sectional area, total lean mass, and lean mass of the legs. pQCT and DXA bone measurements correlate strongly as well, as we see in correlations between trabecular density, cortical area and cortical thickness, and total BMD. The different muscle measurements of pQCT and DXA correlate strongly as well, as we see strong correlations between muscle CSA and total lean mass and muscle CSA and lean mass of the legs.

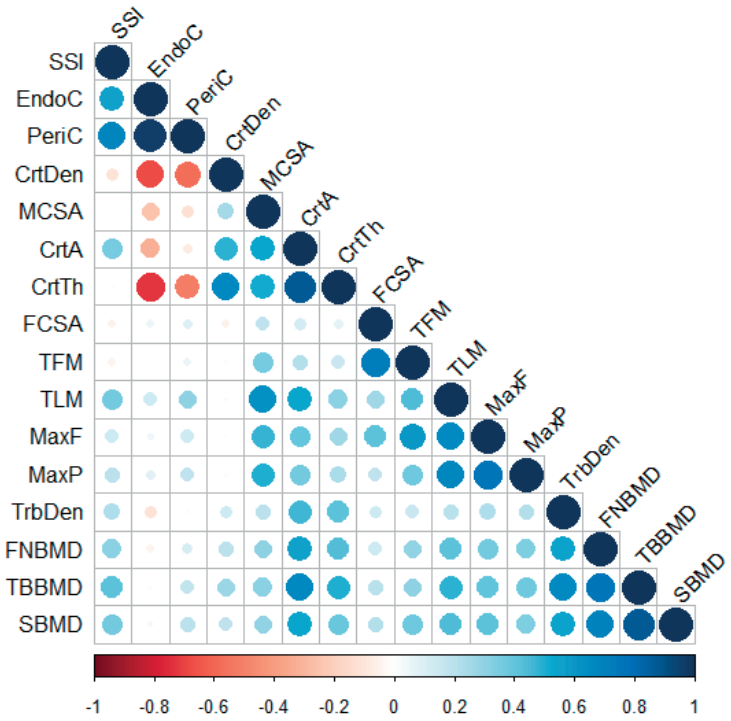


Figure 1 | Genetic correlation between variety of pQCT and DXA parameters. SSI= strength-strain index; endoC= endosteal circumference; PeriC= periosteal circumference; CrtDen= Cortical density; crtA=Cortical area; CrtTh= cortical thickness; trbDen=trabecular density; MCSA = muscle cross sectional area ; FCSA= fat cross sectional area; MaxF= maximal force ; maxP= maximal power; FNBMD=Femoral neck bone mineral density; LSBMD= Lumbar Spine bone mineral density; TBBMD= total body bone mineral density; TFM= total fat mass; TLM= total lean mass.

Table 2 | Association between pQCT and DXA parameters with muscle force and power

N=568	Maximum Force		Maximum Force		Maximum Power		Maximum Power	
	Model 1		Model 2		Model 1		Model 2	
	Beta (95%CI)	P	Beta (95%CI)	P	Beta (95%CI)	P	Beta (95%CI)	P
pQCT parameters								
Trabecular density 4%	0.264 (0.167, 0.361)	< .001	0.174 (0.033, 0.315)	0.012	0.319 (0.196, 0.443)	< .001	0.196 (0.045, 0.347)	0.011
Cortical Area 66%	0.363 (0.291, 0.435)	< .001	0.192 (0.088, 0.295)	< .001	0.438 (0.345, 0.530)	< .001	0.240 (0.129, 0.350)	<0.001
Cortical density 66%	0.005 (-0.086,0.096)	0.914	0.006 (-0.127, 0.139)	0.926	-0.022 (-0.137, 0.093)	0.707	-0.039 (-0.182, 0.104)	0.591
Cortical thickness	0.296 (0.206, 0.385)	< 0.001	0.077 (0.047, 0.306)	0.008	0.328 (0.214, 0.443)	< .001	0.169 (0.029, 0.308)	0.018
Endosteal circumference	0.043 (-0.051,0.137)	0.369	-0.022 (-0.159, 0.115)	0.753	0.110 (-0.008, 0.229)	0.069	0.085 (-0.062, 0.232)	0.259
Periosteal circumference	0.150 (0.067, 0.233)	<0.001	0.037 (-0.085, 0.158)	0.553	0.237 (0.132, 0.342)	< .001	0.153 (0.023, 0.282)	0.021
Muscle CSA	0.471 (0.399, 0.543)	<0.001	0.179 (0.079, 0.280)	<0.001	0.638 (0.547, 0.728)	< .001	0.369 (0.265, 0.474)	<0.001
Fat CSA	0.394 (0.320, 0.467)	<0.001	-0.080 (-0.172, 0.013)	0.091	0.220 (0.120, 0.321)	< .001	-0.326 (-0.422, -0.231	<0.001
SSIp	0.138 (0.064, 0.213)	<0.001	0.083 (-0.023, 0.192)	0.136	0.225 (0.131, 0.319)	< .001	0.180 (0.064, 0.296)	<0.001
DXA parameters								
Total Lean Mass	0.454 (0.410, 0.498)	<0.001	0.153 (0.082, 0.188)	<0.001	0.598 (0.543, 0.652)	< .001	0.305 (253, 0.357)	<0.001
Total Fat	0.617 (0.549, 0.685)	<0.001	-0.115 (-0.167, -0.062)	<0.001	0.482 (0.382, 0.583)	< .001	-0.295 (-0.346, -0.243)	<0.001
Arms Lean Mass	0.345 (0.297, 0.393)	<0.001	0.109 (0.043, 0.174)	<0.001	0.497 (0.439, 0.555)	< .001	0.300 (0.234, 0.336)	<0.001
Legs Lean Mass	0.564 (0.514, 0.613)	<0.001	0.190 (0.132, 0.248)	<0.001	0.745 (0.684, 0.805)	< .001	0.401 (0.346, 0.456)	<0.001
Total BMD	0.411 (0.333, 0.489)	<0.001	0.169 (0.058, 0.280)	0.003	0.485 (0.384, 0.585)	< .001	0.221 (0.103, 0.340)	<0.001
Right Femur BMD	0.317 (0.246, 0.388)	<0.001	0.133 (0.031, 0.234)	0.01	0.405 (0.315, 0.495)	< .001	0.221 (0.113, 0.329)	<0.001
Spine BMD	0.448 (0.364, 0.531)	<0.001	0.147 (0.029, 0.264)	0.014	0.459 (0.350, 0.569)	< .001	0.111 (-0.015, 0.238)	0.084
Right femoral neck BMD	0.392 (0.307,0 .477)	<0.001	0.164 (0.042, 0.285)	0.009	0.461 (0.351, 0.570)	< .001	0.215 (0.084, 0.345)	0.001

Model 1 is adjusted for age and sex

Model 2 is adjusted for age, sex, and weight

Beta-coefficients represent changes in standard deviation for 1 standard deviation increase in maximum force (1SD = 0.36 kN) and maximum power (1SD = 0.87)

Associations of Muscle and Bone

Maximum force and power both showed strong positive associations with trabecular density (force: $\beta = 0.264$, $p < .001$; power: $\beta = 0.319$, $p < .001$), cortical area (force: $\beta = 0.363$, $p < .001$; power: $\beta = 0.438$, $p < .001$), cortical thickness (force: $\beta = 0.296$, $p < .001$; power: $\beta = 0.328$, $p < .001$), periosteal circumference (force: $\beta = 0.150$, $p < .001$; power: $\beta = 0.237$, $p < .001$) and SSI (force: $\beta = 0.138$, $p < .001$; force: $\beta = 0.225$, $p < .001$), but not with cortical density (force: $\beta = 0.005$, $p = 0.914$; force: $\beta = -0.022$, $p = 0.707$) and endosteal circumference (force: $\beta = 0.043$, $p = .369$; power: $\beta = 0.110$, $p = 0.069$), adjusted for age and sex. Various BMD measurements from DXA showed similar associations with both maximum force and power, with slightly larger effects (**Table 2**). Most significant associations remained after added adjustment for weight, although less strong. Changes in significance were observed between maximum force and periosteal circumference ($\beta = 0.037$, $p = 0.553$), maximum force and SSI ($\beta = 0.083$, $p = 0.136$) and maximum power and BMD of the spine ($\beta = 0.111$, $p = 0.084$).

DISCUSSION

Jumping mechanography, pQCT and DXA were used to determine associations between different muscle and bone parameters. pQCT and DXA bone parameters showed similar associations with both power and force, in addition to strong correlations between all parameters. Trabecular density, cortical area and cortical thickness were all strongly associated with maximum force and power. This points to jumping mechanography as a possible predictor of bone health and geometry. Surprisingly, differences in muscle function were not able to predict changes in cortical density. Changes in periosteal circumferences were best explained by changes in power, in contrast to changes in force. The combination endosteal and periosteal circumference, reflected in cortical thickness, was well predicted by both force and power.

A handful of studies have taken a similar approach to investigate the muscle bone relationship. Our results are consistent with previous studies comparing pQCT with jumping mechanography. Similar positive associations between power/force and cortical area have been found in a group of middle-aged adults containing high bone mass individuals as well as control. [23] In a group of 25 to 45-year-old men, power and force have been shown to associate positively with cortical bone, cortical thickness and SSI as well. [25] Furthermore, in a population of 8 to 82-year olds, Anliker et al. [16] found a positive association between maximum force during a m1LH test and volumetric Bone Mineral Content (vBMC) at the multiple sites. Even though this study is in line with our findings, their use of vBMC as opposed to vBMD restricts direct comparisons. Verroken et al. [25] found lean mass of the legs to be the best predictor

of bone characteristics when compared to force, power, MCSA and quadriceps torque. Although the study mentions that only force, not lean mass of the legs, is associated with cortical bone area over total bone area, suggesting force as the best predictor of explicitly cortical bone parameters. Using structural measurements as a predictor of bone characteristics was outside the scope of our study as we decided to focus on the functional muscle parameters provided by JM. Additionally, Rantalainen et al. has found positive associations between maximum jumping force during 2-legged countermovement jump and SSI in pre- and postmenopausal women. [37] These results are very similar to ours in a group of comparable age. In a group of pre-pubertal females and males SSI has been shown to correlate most strongly with power, and force to a lesser degree. [38] Although comparing this with our study might be of limited value due to the enormous difference in age. Whether force or power is more useful remains unclear as sometimes power, [23,38] sometimes force [25] is the better predictor of bone parameters.

In the current study, power was a slightly better predictor for most pQCT and DXA variables, although not statically significant. This difference might originate in our use of a suboptimal test to assess maximum force. As mentioned before, the m1LH test is more suitable for maximum force while a s2LJ is more suitable of assessing maximum power. The s2LJ might underestimate maximum force as discussed by Anliker et al. [16] This underestimation might help explain the difference between this study and other studies.

Moreover, we found that both cortical parameters as well as trabecular density are predicted by force. As cortical geometry seems to be the most important predictor of future fracture, [39-41] pQCT derived cortical thickness and SSI may be useful measurements in identifying at risk individuals. On the other hand, metabolic activity in bone is the highest in the trabecular bone. vBMD measurements might subsequently be used to identify the first signs of decline in bone health. Whether changes in cortical geometry really trail trabecular changes cannot be researched in a cross-sectional design study. Repeated measurements and an increase of sample size in the current cohort could possibly be used for these purposes.

Even though peak muscle forces are believed to be the main force behind changes in bone geometry, the effect of weight might still account for some portion of the changes by putting a load on weight bearing bones.[42-45] As we see in the differences before and after weight adjustment, weight seems to account for a non-negligible portion of the changes in bone geometry. During regression analysis (not shown here) of normal and high BMI groups, we found the jumping mechanograph to predict bone health less well in the high BMI group, as opposed to the normal BMI group. The lower coefficients in the high BMI group expose weight as a mediating factor when predicting bone health with a jumping mechanograph. A rational explanation would be that

with the increases in weight, the weight takes an increasing role in bone geometry due to increases in gravitational loading as opposed to muscle loading. While muscle contractions are believed to be the main cause of changes in bone, weight seems to account for a bigger part of the changes in high BMI individuals as opposed to normal BMI individuals. The importance of weight on bone geometry might be more significant in high BMI individuals, but more research needs to be done to confirm this.

To our knowledge, the present study provides the largest population to investigate the bone-muscle relationship using both pQCT and jumping mechanography. In addition, our population contains both male and female participants in contrast to most other studies. Another advantage of our study is the use of multiple methods of assessing bone characteristics. pQCT is a very accurate way to investigate bone and show different aspects, DXA on the other hand has long been a household method of investigating bone. We reported both with and without weight adjustment to be complete and because most comparable studies did adjust for weight.

We do recognize some limitations in the current study as well. First of all, menopause status has not been used for women. As the menopause has a substantial effect on bone geometry, the absence of information in this regard limits drawing of conclusions. Secondly, we used a single 2-legged jump to assess maximum force. As mentioned before, this type of jump is most suitable in the assessment of maximum power. A multiple 1-legged hopping test would be better suited to assess maximum force. Thirdly, our study population can be considered too young to truly investigate the decline of bone health in relation to muscle function as our population is in the beginning stages of the decline. Still, this study does show the association between the two with less focus on the pathological process. We are missing physical activity so this can be limitation also given it an important predictor of both power and bone.

These findings help us understand the possible mechanisms of changes in bone geometry and might help in the early recognition of bone deterioration. Not only that, the strong link between bone and muscle also provides a possible solution to prevent further decline in both health by encouraging regular exercise with a focus on muscle strength.

In conclusion, our study has demonstrated the ability of the jumping mechanography in explaining variation in bone parameters as measured by pQCT and DXA. In combination with other studies, this solidifies jumping mechanography as a good, low burden tool in the assessment of bone health. In addition, pQCT has been demonstrated as a valuable alternative to DXA. Even though jumping mechanography predicts both pQCT and DXA approximately equally well, the ability of pQCT to deliver more in-depth information about bone geometry is highly valuable in the investigation of underlying mechanisms.

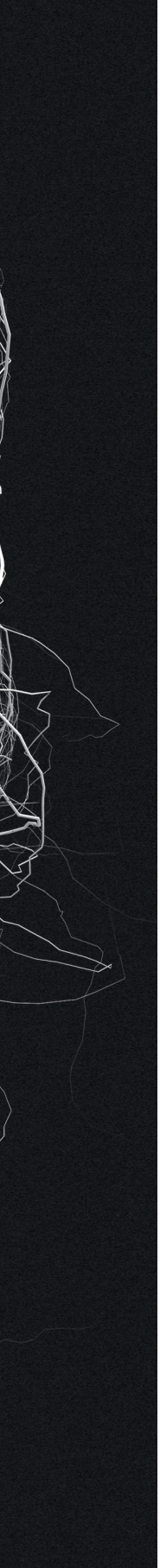
REFERENCES

1. Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. BMJ. 1996;312(7041):1254-9.
2. Cummings SR, Black DM, Nevitt MC, Browner W, Cauley J, Ensrud K, et al. Bone density at various sites for prediction of hip fractures. The Study of Osteoporotic Fractures Research Group. Lancet. 1993;341(8837):72-5.
3. Abrahamsen B, van Staa T, Ariely R, Olson M, Cooper C. Excess mortality following hip fracture: a systematic epidemiological review. Osteoporos Int. 2009;20(10):1633-50.
4. Katsoulis M, Benetou V, Karapetyan T, Feskanich D, Grodstein F, Pettersson-Kymmer U, et al. Excess mortality after hip fracture in elderly persons from Europe and the USA: the CHANCES project. J Intern Med. 2017;281(3):300-10.
5. Haentjens P, Magaziner J, Colon-Emeric CS, Vanderschueren D, Milisen K, Velkeniers B, et al. Meta-analysis: excess mortality after hip fracture among older women and men. Ann Intern Med. 2010;152(6):380-90.
6. Ferretti JL, Capozza RF, Cointry GR, Garcia SL, Plotkin H, Alvarez Filgueira ML, et al. Gender-related differences in the relationship between densitometric values of whole-body bone mineral content and lean body mass in humans between 2 and 87 years of age. Bone. 1998;22(6):683-90.
7. Compston JE, Bhambhani M, Laskey MA, Murphy S, Khaw KT. Body composition and bone mass in post-menopausal women. Clin Endocrinol (Oxf). 1992;37(5):426-31.
8. Tsunenari T, Tsutsumi M, Ohno K, Yamamoto Y, Kawakatsu M, Shimogaki K, et al. Age- and gender-related changes in body composition in Japanese subjects. J Bone Miner Res. 1993;8(4):397-402.
9. Bauer DC, Browner WS, Cauley JA, Orwoll ES, Scott JC, Black DM, et al. Factors associated with appendicular bone mass in older women. The Study of Osteoporotic Fractures Research Group. Ann Intern Med. 1993;118(9):657-65.
10. Nguyen TV, Center JR, Eisman JA. Osteoporosis in elderly men and women: effects of dietary calcium, physical activity, and body mass index. J Bone Miner Res. 2000;15(2):322-31.
11. Snow-Harter C, Bouxsein M, Lewis B, Charette S, Weinstein P, Marcus R. Muscle strength as a predictor of bone mineral density in young women. J Bone Miner Res. 1990;5(6):589-95.
12. Schiessl H, Frost HM, Jee WS. Estrogen and bone-muscle strength and mass relationships. Bone. 1998;22(1):1-6.
13. Frost HM. Bone's mechanostat: a 2003 update. Anat Rec A Discov Mol Cell Evol Biol. 2003;275(2):1081-101.
14. Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 1. Redefining Wolff's law: the bone modeling problem. Anat Rec. 1990;226(4):403-13.
15. Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 2. Redefining Wolff's law: the remodeling problem. Anat Rec. 1990;226(4):414-22.
16. Anliker E, Rawer R, Boutellier U, Toigo M. Maximum ground reaction force in relation to tibial bone mass in children and adults. Med Sci Sports Exerc. 2011;43(11):2102-9.
17. Anliker E, Toigo M. Functional assessment of the muscle-bone unit in the lower leg. J Musculoskelet Neuronal Interact. 2012;12(2):46-55.
18. WHO. Prevention and management of osteoporosis. World Health Organ Tech Rep Ser. 2003;921:1-164, back cover.

19. Amstrup AK, Jakobsen NF, Moser E, Sikjaer T, Mosekilde L, Rejnmark L. Association between bone indices assessed by DXA, HR-pQCT and QCT scans in post-menopausal women. J Bone Miner Metab. 2016;34(6):638-45.
20. Stagi S, Cavalli L, Cavalli T, de Martino M, Brandi ML. Peripheral quantitative computed tomography (pQCT) for the assessment of bone strength in most of bone affecting conditions in developmental age: a review. Ital J Pediatr. 2016;42(1):88.
21. Nishiyama KK, Shane E. Clinical imaging of bone microarchitecture with HR-pQCT. Curr Osteoporos Rep. 2013;11(2):147-55.
22. Guerri S, Mercatelli D, Aparisi Gomez MP, Napoli A, Battista G, Guglielmi G, et al. Quantitative imaging techniques for the assessment of osteoporosis and sarcopenia. Quant Imaging Med Surg. 2018;8(1):60-85.
23. Hardcastle SA, Gregson CL, Rittweger J, Crabtree N, Ward K, Tobias JH. Jump power and force have distinct associations with cortical bone parameters: findings from a population enriched by individuals with high bone mass. J Clin Endocrinol Metab. 2014;99(1):266-75.
24. Buehring B, Krueger D, Binkley N. Jumping mechanography: a potential tool for sarcopenia evaluation in older individuals. J Clin Densitom. 2010;13(3):283-91.
25. Verroken C, Zmierczak HG, Goemaere S, Kaufman JM, Lapauw B. Association of Jumping Mechanography-Derived Indices of Muscle Function with Tibial Cortical Bone Geometry. Calcif Tissue Int. 2016;98(5):446-55.
26. Ashe MC, Liu-Ambrose TY, Cooper DM, Khan KM, McKay HA. Muscle power is related to tibial bone strength in older women. Osteoporos Int. 2008;19(12):1725-32.
27. Zengin A, Pye SR, Cook MJ, Adams JE, Rawer R, Wu FCW, et al. Associations of muscle force, power, cross-sectional muscle area and bone geometry in older UK men. J Cachexia Sarcopenia Muscle. 2017;8(4):598-606.
28. Nilsson M, Sundh D, Mellstrom D, Lorentzon M. Current Physical Activity Is Independently Associated With Cortical Bone Size and Bone Strength in Elderly Swedish Women. J Bone Miner Res. 2017;32(3):473-85.
29. Edwards MH, Gregson CL, Patel HP, Jameson KA, Harvey NC, Sayer AA, et al. Muscle size, strength, and physical performance and their associations with bone structure in the Hertfordshire Cohort Study. J Bone Miner Res. 2013;28(11):2295-304.
30. Cousins JM, Petit MA, Paudel ML, Taylor BC, Hughes JM, Cauley JA, et al. Muscle power and physical activity are associated with bone strength in older men: The osteoporotic fractures in men study. Bone. 2010;47(2):205-11.
31. Kaji H, Kosaka R, Yamauchi M, Kuno K, Chihara K, Sugimoto T. Effects of age, grip strength and smoking on forearm volumetric bone mineral density and bone geometry by peripheral quantitative computed tomography: comparisons between female and male. Endocr J. 2005;52(6):659-66.
32. Elhakeem A, Hartley A, Luo Y, Goertzen AL, Hannam K, Clark EM, et al. Lean mass and lower limb muscle function in relation to hip strength, geometry and fracture risk indices in community-dwelling older women. Osteoporos Int. 2019;30(1):211-20.
33. Veilleux LN, Rauch F. Reproducibility of jumping mechanography in healthy children and adults. J Musculoskelet Neuronal Interact. 2010;10(4):256-66.
34. Matheson LA, Duffy S, Maroof A, Gibbons R, Duffy C, Roth J. Intra- and inter-rater reliability of jumping mechanography muscle function assessments. J Musculoskelet Neuronal Interact. 2013;13(4):480-6.

35. Trajanoska K, Schoufour JD, de Jonge EAL, Kieboom BCT, Mulder M, Stricker BH, et al. Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. Bone. 2018;114:116-24.
36. Ikram MA, Brusselle GGO, Murad SD, van Duijn CM, Franco OH, Goedegebure A, et al. The Rotterdam Study: 2018 update on objectives, design and main results. Eur J Epidemiol. 2017;32(9):807-50.
37. Rantalainen T, Nikander R, Heinonen A, Multanen J, Hakkinen A, Jamsa T, et al. Neuromuscular performance and body mass as indices of bone loading in premenopausal and postmenopausal women. Bone. 2010;46(4):964-9.
38. Binkley TL, Specker BL. Muscle-bone relationships in the lower leg of healthy pre-pubertal females and males. J Musculoskelet Neuronal Interact. 2008;8(3):239-43.
39. Dennison EM, Jameson KA, Edwards MH, Denison HJ, Aihie Sayer A, Cooper C. Peripheral quantitative computed tomography measures are associated with adult fracture risk: the Hertfordshire Cohort Study. Bone. 2014;64:13-7.
40. Sheu Y, Zmuda JM, Boudreau RM, Petit MA, Ensrud KE, Bauer DC, et al. Bone strength measured by peripheral quantitative computed tomography and the risk of nonvertebral fractures: the osteoporotic fractures in men (MrOS) study. J Bone Miner Res. 2011;26(1):63-71.
41. Stein EM, Kepley A, Walker M, Nickolas TL, Nishiyama K, Zhou B, et al. Skeletal structure in postmenopausal women with osteopenia and fractures is characterized by abnormal trabecular plates and cortical thinning. J Bone Miner Res. 2014;29(5):1101-9.
42. Robling AG. Is bone's response to mechanical signals dominated by muscle forces? Med Sci Sports Exerc. 2009;41(11):2044-9.
43. Judex S, Carlson KJ. Is bone's response to mechanical signals dominated by gravitational loading? Med Sci Sports Exerc. 2009;41(11):2037-43.
44. Kohrt WM, Barry DW, Schwartz RS. Muscle forces or gravity: what predominates mechanical loading on bone? Med Sci Sports Exerc. 2009;41(11):2050-5.
45. Hart NH, Nimphius S, Rantalainen T, Ireland A, Siafarikas A, Newton RU. Mechanical basis of bone strength: influence of bone material, bone structure and muscle action. J Musculoskelet Neuronal Interact. 2017;17(3):114-39.





3.2

Sarcopenia and Its Clinical Correlates in the General Population: The Rotterdam Study

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ABSTRACT

Sarcopenia, a complex multifactorial condition, is characterized by loss of muscle mass and function, which increases progressively with age. The existence of different definitions has contributed to the large variation in the prevalence estimates of sarcopenia. We aimed to estimate the prevalence of sarcopenia in the general population using the European Working Group on Sarcopenia in Older People (EWGSOP) proposed definition and compared baseline demographic and clinical characteristics between the nonsarcopenia, presarcopenia, and sarcopenia individuals, with particular emphasis on the overlap with osteoporosis and fracture risk. We studied 5911 subjects at a mean age of 69.2 years (55.8% female) with data on sarcopenia participating in the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. Presarcopenia was defined as having only low muscle mass, whereas sarcopenia was defined based on the presence of low muscle mass, plus either low muscle strength or low physical performance. The prevalence of presarcopenia and sarcopenia was 5.9% and 4.4%, respectively. Individuals with sarcopenia were older, more often males, smokers, with less optimal dietary intake, and more often disabled with lower physical activity. Although the prevalence of fractures was higher in individuals with low lean mass (presarcopenic [16.6%] and sarcopenic [23.5%]) compared with the no sarcopenic group (15.5%), the differences were not present after correcting for age and sex. There were no statistical differences in the prevalence of chronic diseases, with the exception of a higher prevalence of COPD in presarcopenic (29.1%) and sarcopenic (26.9%) individuals compared with nonsarcopenic (13.4%) individuals. Osteoporotic individuals with (odds ratio [OR] = 2.59, 95% confidence interval [CI] 1.4-4.45) and without sarcopenia (OR = 2.75, 95% CI 2.0-3.75) had similar elevated risk of nonvertebral fractures. The presence of sarcopenia appears to be independent of chronic diseases with the exception of COPD and more related to lifestyle factors and disabilities. Sarcopenic individuals in the general population are at no greater risk of fracture than what is determined by their low bone mineral density.

INTRODUCTION

Sarcopenia, the loss of muscle mass with age, has been postulated as one of the most important geriatric syndromes. [1] The prevalence of sarcopenia has been estimated to range from 1% to 33%,[2-6] depending on the population settings and the assessment techniques and cut-offs used. Sarcopenia contributes to functional decline, falls, morbidity, and mortality of elderly people and imposes a high cost to the health care system. [7-9] Furthermore, it is considered to precede physical frailty[10] and, as such, an important pathway through which the adverse associated health outcomes of frailty develop. [11] The increase in life expectancy and growth of the elderly population will likely result in a growing prevalence of the condition and its associated adverse health outcomes.

In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) provided a practical clinical and consensus definition, which constitutes the most commonly used diagnostic criteria of sarcopenia.[1] For the diagnosis of sarcopenia, a combination of the presence of low muscle mass and of compromised low muscle function (strength or performance) is required.[1] However, very few studies have applied the EWGSOP criteria in the general population on sufficient scale to obtain stable prevalence estimates. Moreover, little knowledge exists surrounding the relationship between sarcopenia (using the new consensus definition) and its associated factors within large population-based studies. Identifying modifiable risk factors for sarcopenia will help to establish evidence-based interventions to prevent the onset of sarcopenia and associated conditions in order to extend the time free from disability in older adults. Last but not least, sarcopenia has been associated with increased fracture risk. [12] However, sarcopenia may co-occur with osteoporosis, a condition referred to as osteosarcopenia, and it is not clear if the effect of sarcopenia is independent of the bone mineral density (BMD) status. Therefore, our aim was to 1) investigate the prevalence of sarcopenia in the general population using the EWGSOP proposed definition, cut-offs, and measures; 2) identify factors related to sarcopenia by comparing baseline demographic and clinical characteristics between the nonsarcopenia, presarcopenia, and sarcopenia individuals; and 3) estimate if individuals with sarcopenia and osteoporosis have greater risk of fractures compared with individuals with sarcopenia or osteoporosis alone.

MATERIALS AND METHODS

Study Population

Our study included individuals from the Rotterdam Study,[13] an ongoing population-based prospective cohort comprising almost 15,000 Dutch individuals aged 45 years and older. After its start in 1990, a total of 7983 people were included in the initial study wave (RSI). In 2000 and 2006, the cohort was expanded with 3011 (RSII) and 3932 participants (RSIII), respectively. All participants underwent an extensive home interview followed by a visit to the research center where various physical and laboratory examinations took place, all following standardized protocols. Every 2 to 4 years, the participants were invited for follow-up visits. The Rotterdam Study was approved by the medical ethics committee of the Erasmus MC and by the Ministry of Health, Welfare, and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All individuals provided written informed consent to participate in the study and to retrieve information from their treating physicians. The present study used data assessed at the fifth visit of the first Rotterdam Study cohort (RSI-5, 2009–11; n =1521), the third visit of the second cohort (RSII-3, 2011–12; n =1603), and the second visit of the third cohort (RSIII-2, 2012–014; n =2787), for a total of 5911 participants with complete information.

Assessment of Sarcopenia

We used the EWGSOP definition to define sarcopenia as the presence of low muscle mass, plus either low muscle strength or low physical performance (**Figure 1**). Presarcopenia was defined as just having low muscle mass without compromising muscle function or physical performance.[1]

Lean mass was measured using dual-energy X-ray absorptiometry (DXA) with an iDXA total body fan-beam densitometer (GE Lunar Corp., Madison, WI, USA). The scans were analyzed using the enCORE software that employs an algorithm, which divides the total body by regions of interest into head, trunk, arms, and legs. Appendicular lean mass (ALM) is the sum of the lean tissue from the arms and legs. Skeletal muscle index (SMI), adjusted for variation in skeletal size, was defined as ALM divided by squared body height (kg/m^2). Low muscle mass was defined as SMI ≤ 7.25kg/m^2 for males and ≤ 5.67 kg/m^2 for females. [14] Muscle strength was assessed using a hydraulic hand dynamometer (Fabrication Enterprises Inc., White Plains, NY, USA) and maximum grip strength was defined as the maximum value (kg) out of the three trials performed in the nondominant hand. [13] Low muscle strength was defined differentially across sexes and according to body mass index (BMI) levels. In men, as a handgrip of ≤ 29kg (if BMI ≤ 24), ≤ 30kg (if BMI ≤ 24.1–28), or 3≤ 2kg (if BMI >28). In women, ≤ 17kg (if BMI ≤ 23), ≤ 17.3kg (if BMI ≤23.1–26), ≤ 18 kg (if BMI ≤ 26.1–29), or ≤ 21kg (if BMI>29).

[15] Physical performance was assessed as gait speed during normal walk using the GAITRite walkway (CIR Systems, Inc., Sparta, NJ, USA), a 5.79-m-long electronical walkway. [16,17] Low physical performance was defined differentially across sexes and according to body height: in men, as a gait speed <0.65 m/s (if height ≤ 173 cm) or <0.76 m/s (if height >173 cm), and in women, as having a gait speed <0.65 m/s (if height ≤ 159 cm) or <0.76 m/s (if height >159 cm).(15) Using these criteria, individuals were classified into one of three groups: 1) sarcopenic; 2) presarcopenic; and 3) non-sarcopenic (**Figure 1**).

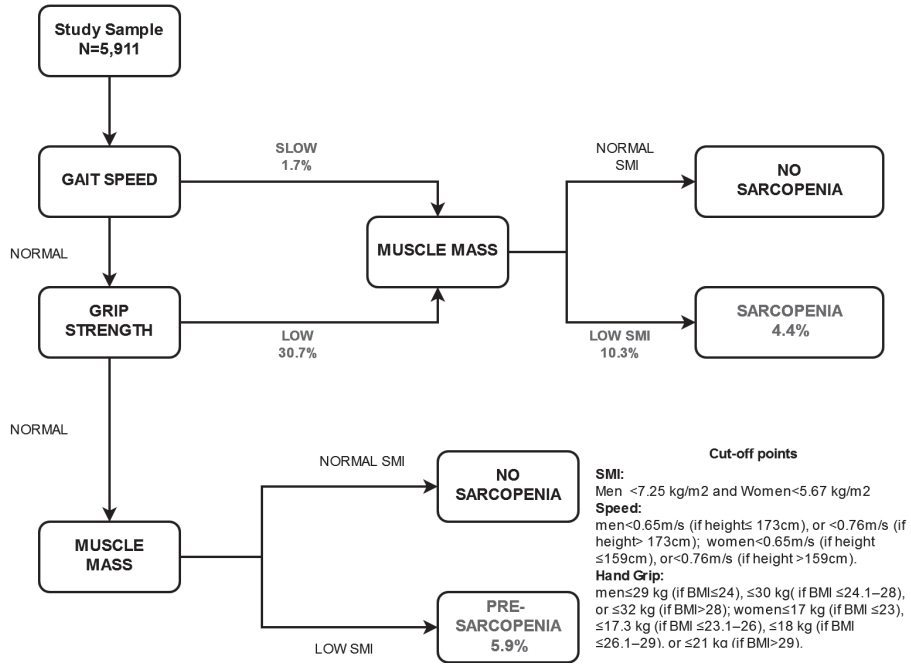


Figure 1 | Application of the European Working Group on Sarcopenia in Older People algorithm

Assessment of Bone Mineral Density

Femoral neck BMD was measured using dual-energy X-ray absorptiometry (DXA) with the same iDXA total body fan-beam densitometer (GE Lunar Corp.). Sex-specific T-scores were calculated using the NHANES III reference population. [18] Osteosarcopenia was defined as sarcopenia plus low BMD (T-score <-1 SD).

Assessment of Associated Factors

Height (cm) and weight (kg) were measured in the research center with the individuals in standing position wearing indoor clothing without shoes. Body mass index was computed as weight in kilograms divided by height in meters squared (kg/m^2). Smok-

ing, education, living status, hospitalization, falls, and pain were collected through self-report. Excessive alcohol consumption was defined as >20 g per day for women and >30 g per day for men. Diet quality was assessed using validated food-frequency questionnaires (FFQ) to assess usual dietary intake in the last month. Next, diet quality was calculated as adherence to the Dutch Dietary Guidelines (2006), as expressed in the Dutch Healthy Diet Index.[19] Physical activity levels were assessed with an adapted version of the LASA Study Physical Activity Questionnaire,[20,21] including questions regarding walking, cycling, sports, gardening, hobbies, and housekeeping. Answers were translated into metabolic equivalent of task (MET) values, and individuals were classified in quartiles. Cognitive function was assessed by the Dutch version of the 30-point Mini-Mental State Examination (MMSE), with a score of 26 or below indicating poor cognition.[22] Disability was assessed using the Stanford Health Assessment Questionnaire (HAQ) that measures disability across eight fields.[23] Mild disability was defined as having difficulties with some of the items and severe as having difficulties with more than half of the items. Prevalent fractures were reported by general practitioners (GPs) in the research area by means of a computerized system or through hospital records. Fracture information from GPs outside the research area was obtained by regular monitoring of the patient record by research physicians. All reported events were verified by a research physician who independently reviewed and coded the information. Metabolic syndrome (MetS) was diagnosed if at least three of the following five traits were present: 1) abdominal obesity, defined as waist circumference >102 cm in men and >88 cm in women; 2) serum triglycerides ≥ 1.7 mmol/L or drug treatment for elevated triglycerides; 3) serum high-density lipoprotein cholesterol (HDL-C) <1.0 mmol/L in men and <1.3 mmol/L in women or drug treatment for low HDL-C; 4) blood pressure ≥ 130/85 mmHg or drug treatment for elevated blood pressure; and 5) fasting plasma glucose (FPG) ≥ 100 mg/ dL (5.6 mmol/L) or drug treatment for elevated blood glucose. Coronary heart disease was defined as coronary revascularization or myocardial infarction or death due to coronary heart disease.[24] Diabetes mellitus (DM) was defined from combining information on glucose-lowering medication use, blood glucose levels, and diagnosis in the GP registries. [25] Chronic obstructive pulmonary disease (COPD) was assessed by spirometry (FEV1/ FVC <0.70), performed at the research center or, if not available, confirmed by a physician based on the combination of clinical history, physical examination, and spirometry con outside the research center.[26] Stroke was defined as syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function with symptoms lasting 24 hours or longer or leading to death, with no apparent origin other than vascular.[27] Depression was defined using the CES-D Scale (Center for Epidemiology Studies Depression Scale), with a score of 16 or higher indicating the presence of depressive symptoms.[28] History of neoplasms was collected through self-report.

Biochemistry

Serum cholesterol, triglycerides, glucose, insulin, serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase, gamma-glutamyltransferase (GGT), alkaline phosphatase, hemoglobulin, and total bilirubin were measured using automatic enzyme procedures. Homeostasis model assessment of insulin resistance (HOMA-IR) was used as proxy for insulin resistance and calculated by multiplying fasting glucose (mmol/L) by fasting insulin (mU/L) divided by 22.5.

STATISTICAL ANALYSES

Characteristics for the population were provided for nonsarcopenic, presarcopenic and sarcopenic individuals. Continuous variables are reported as mean SD unless stated otherwise, and categorical variables were presented as percentages. Differences between the nonsarcopenic and sarcopenic individuals in demographic and clinical characteristics were assessed with crude and age-, sex-, and cohort-adjusted linear and logistic regression models. The same approach was done comparing sarcopenia only, osteoporosis only, and osteosarcopenia. Logistic regression analysis was used to calculate the fracture risk odds ratio (ORs) and 95% confidence intervals (CIs) per group (osteopenia and no [pre] sarcopenia; osteoporosis and no [pre] sarcopenia; normal bone and presarcopenia; osteopenia and presarcopenia; osteoporosis and presarcopenia; normal bone and sarcopenia; osteopenia and sarcopenia; osteoporosis and sarcopenia) using the participants with no low bone mineral density and no sarcopenia as reference. All statistical analyses were performed using R (version 3.3.1). Any p values <0.05 were considered statistically significant.

As sensitivity, we compared the sarcopenia prevalence estimate from the EWGSOP definition with the estimate derived by the Foundation of the National Institutes of Health Sarcopenia Project (FNIH) definition and cut-off points. Moreover, we tested the association of 1-unit increase in T-score (with and without sarcopenia) with fracture.

RESULTS

Individual demographic and clinical variables are summarized in **Table 1**. The majority of the individuals were classified as nonsarcopenic (89.7%). Prevalence of presarcopenia and sarcopenia was 5.9% and 4.4%, respectively. The cases' overlap between the EWGSOP and FNIH definitions was relatively small (**Supplementary Figure 1**). On average, individuals were 69.2 ± 9.1 years old and 56.8% were female. The mean age of individuals with sarcopenia was higher (78.0 ± 8.5 years) than those without

Table 1 | Characteristics of Study Population Participants According to the Presence of Sarcopenia

Study characteristics	No (pre) sarcopenia	Presarcopenia	Sarcopenia	% Missing
Prevalence, n(%)	5,301 (89.7)	350 (5.9)	260 (4.4)	
Age (years), mean ± SD	68.72 ± 8.9	70.42 ± 8.8	77.98 ± 8.5[a]	-
Female,n(%)	3117 (58.8)	146 (41.7)[a]	98 (37.7)[a]	-
Educational level, n (%)				1
Primary	438 (8.4)	30 (8.6)	25 (9.7)	
Intermediate	3661 (69.8)	223 (64.1)	184 (71.3)	
High	1145 (21.8)	95 (27.3)	49 (19.0)	
DHD index score, mean ± SD	56.41 ± 9.9	55.85 ± 9.9	54.37 ± 11.2	17.3
Alcohol ≥20 g/d (women) or ≥30 g/d (men)	350 (7.7)	25 (8.4)	12 (6.0)	15
Smoking, n (%)				0.2
Never	1735 (32.8)	91 (26.0)	68 (26.3)	
Former	2782 (52.6)	176 (50.3)	148 (57.1)	
Current	771 (14.6)	83 (23.7)[a]	43 (16.6)[a]	
Physical activity, median (IQR)	41.5 (17.0–78.9)	31.5 (13.9–75.9)[a]	19.4 (9.0–47.6)[a]	14.2
Height (cm), mean (SD)	168.6 ± 9.5	171.7 ± 9.6[a]	167.8 ± 9.9[a]	-
BMI (kg/m^2), mean (SD)	28.0 ± 4.1	22.8 ± 2.5[a]	23.7 ± 2.9[a]	-
Normal (BMI 18.5–25)	1226 (23.2)	278 (83.5)	178 (70.0)	
Overweight (BMI 25–30)	2680 (50.6)	54 (16.2)	70 (27.6)	
Obese (BMI 30+)	1387 (26.2)	1 (0.3)	6 (2.4)	
WHR, mean (SD)	0.90 ± 0.1	0.88 ± 0.1[a]	0.91 ± 0.1[a]	0.05
Femoral neck BMD (g/m^2), mean ± SD	0.91 ± 0.1	0.86 ± 0.1[a]	0.83 ± 0.2[a]	4.3
Disability index, n (%)				6.2
No disability	3874 (77.6)	276 (83.1)	119 (54.3)	
Mild disability	883 (17.7)	44 (13.3)	68 (31.1)[a]	
Severe disability	234 (4.7)	12 (3.6)	32 (14.6)[a]	
MMSE, n (%)				0.19
Cognitive impairment (<26)	404 (7.3)	74 (13.4)[a]	45 (18.3)	
No cognitive impairment (≥26)	4886 (92.4)	303 (86.6)	215 (82.7)	
Living status, (%)				0.2
Independent	4,929 (93.2)	318 (90.9)	220 (84.9)	
Other	361 (6.8)	32 (9.1)	39 (15.1)	
Hospitalization. yes.n(%)	203 (14.8)	25 (20.8)	33 (21.3)	72.1
Pain, yes, n(%)	1,403 (71.7)	91 (53.8)[a]	111 (63.1)	61.1
Falls, yes, n (%)	505 (25.8)	43 (25.4)	55 (31.2)	61.1

DHD = Dutch health diet; BMI = body mass index; WHR= waist hip ratio; BMD = bone mineral density; MMSE = Mini-Mental State Examination.

a Significant p value <0.05 comparing no (pre) sarcopenia with presarcopenia and sarcopenia groups adjusted for age, sex, and cohort effect. For the group comparison analyses, physical activity was log transformed.

sarcopenia (68.7 ± 8.9 years). In the presarcopenic and sarcopenic groups, there were more men than women (58.3% and 62.3%, respectively). Overall, there was a marked increase in the prevalence of sarcopenia with age, being particularly prominent in men after the age of 70 years, reaching 22.1% after the age of 80 years. In women, the prevalence of sarcopenia was lower than in men, reaching 9.3% after age 80 years (**Figure 2**).

Presarcopenic and sarcopenic individuals were more often smokers, were more often hospitalized, lived independently less often, and were more disabled than non-sarcopenic individuals. Furthermore, they were less active and had lower diet quality. After controlling for age and sex, these differences remained significant only for physical activity, smoking, and disability status. Moreover, the nonsarcopenia group

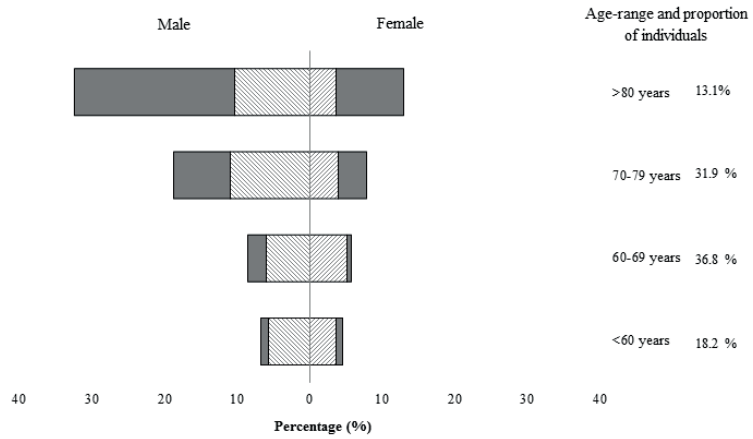


Figure 2 | Prevalence of sarcopenia and presarcopenia by sex and age decades

had higher number of MetS compared with the other groups.

Biochemical characteristics of the study are present in **Table 2**. After age and sex adjustment, the presarcopenia and sarcopenia groups tended to have lower levels of triglycerides, ALT, bilirubin, hemoglobulin, glucose, insulin, and HOMA-IR and higher levels of HDL. Compared with the nonsarcopenic group, individuals with sarcopenia had lower BMD and had higher prevalence of fractures (23.5% versus 15.5%) (**Table 3**). However, the difference in prevalent fractures was driven by age and sex. Furthermore, individuals with sarcopenia had higher prevalence of chronic comorbid conditions, but the differences were not significant with the exception of stroke, coronary heart disease, and COPD. Interestingly, after controlling for age and sex, the prevalence of COPD in the presarcopenia (29.1%) and sarcopenia (26.9%) groups was significantly higher compared with the nonsarcopenia group (13.4%). Notably, the

Table 2 | Biochemical Characteristics of Study Participants According to the Presence of Sarcopenia

Study characteristics	No (pre) sarcopenia	Presarcopenia	Sarcopenia
Cholesterol (mmol/L), mean ± SD	5.5 ± 1.11	5.4 ± 1.04	5.2 ± 1.08
HDL cholesterol (mmol/L), mean ± SD	1.5 ± 0.43	1.6 ± 0.47[a]	1.5 ± 0.43[a]
Triglycerides (mmol/L), mean ± SD	1.5 ± 0.77	1.4 ± 0.73[a]	1.3 ± 0.63[a]
Aspartate transaminase (U/L), median (IQR)	24.0 (21.0–28.0)	24.0 (21.0–28.0)	24.0 (21.0–28.0)
ALT (U/L), median (IQR)	19.0 (15.0–24.0)	17.5 (14.0–23.0)[a]	17.0 (13.0–22.0)[a]
ALP (U/L), median (IQR)	68.0 (58.0–80.0)	68.5 (57.0–83.0)	69.5 (59.0–85.3)[a]
Bilirubin (µmol/L), median (IQR)	8.0 (6.0–11.0)	8.0 (6.0–11.0)	8.0 (6.0–10.3)[a]
GGT (U/L), median (IQR)	24.0 (17.0–35.0)	24.0 (17.0–33.0)	23.0 (17.0–37.0)
Creatinine (µmol/L), median (IQR)	77.0 (67.0–90.0)	77.0 (67.0–90.0)[a]	80.0 (69.0–97.0)
Hemoglobulin (g/dL), mean ± SD	8.8 ± 0.8	8.6 ± 0.8	8.5 ± 0.9[a]
Glucose (mmol/L), median (IQR)	5.5 (5.1–6.1)	5.3 (5.0–5.80)[a]	5.4 (5.10–6.1)[a]
Insulin (pmol/L), median (IQR)	75.0 (53.0–109.0)	55.0 (39.0–80.0)[a]	63.5 (44.00–89.0)[a]
HOMA-IR, median (IQR)	2.7 (1.78–4.17)	1.9 (1.3–2.8)[a]	2.2 (1.47–3.3)[a]

HDL= high-density lipoprotein; ALT = alanine aminotransferase; ALP = alkaline phosphatase; GGT = gamma-glutamyl transferase; HOMA-IR = homeostatic model assessment-insulin resistance.
a Significant p value (<0.05) comparing no (pre) sarcopenia with presarcopenia and sarcopenia groups adjusted for age, sex, and cohort effect. For the group comparison analyses, the variables presented as median (IQR) were log transformed.

Table 3 | Clinical Characteristics of Study Participants According to the Presence of Sarcopenia

Study characteristics	No (pre) sarcopenia	Presarcopenia	Sarcopenia	% Missing
Prevalence, n (%)	5301 (89.7)	350 (5.9)	260 (4.4)	
History of non-vertebral fracture, n (%)	820 (15.5)	58 (16.6)	61 (23.5)	-
Metabolic syndrome, n (%)	1768 (34.0)	50 (14.5)[a]	41 (16.5)[a]	2.1
Blood pressure >130/85 mmHg	3964 (74.9)	246 (70.3)[a]	207 (80.2)[a]	1.9
Coronary heart disease, n (%)	187 (3.6)	11 (3.2)	19 (7.5)	0.7
Diabetes mellitus, n (%)	477 (9.1)	17 (4.9)[a]	28 (11.0)	1.4
COPD, n (%)	710 (13.4)	102 (29.1)[a]	70 (26.9)[a]	2
Stroke, n (%)	184 (3.5)	13 (3.7)	20 (7.7)	-
Neoplasm, n (%)	210 (15.3)	15 (12.4)	31 (20.3)	72.1
Depression, n (%)	405 (8.0)	29 (8.8)	21 (9.1)	4.6

COPD = chronic obstructive pulmonary disease.
a Significant p value (<0.05) comparing no (pre) sarcopenia with presarcopenia and sarcopenia groups adjusted for age, sex, and cohort effect.

association with COPD remained significant after additional controlling for smoking, physical activity, and number of prescribed corticosteroids.

We then moved on to compare sarcopenic individuals according to osteoporosis status. Individuals with sarcopenia only (n = 47) had similar clinical and biochemical characteristics compared with the individuals with osteoporosis only (n = 278) and osteosarcopenia (n = 195) (**Table 4**). Individuals with osteoporosis only had higher

prevalence of fractures (34.5%) compared with individuals with sarcopenia only (17.0%) (**Table 5**). The sarcopenia only group compared with the osteosarcopenia

Table 4 | Comparing Demographic and Clinical Characteristics Between Sarcopenia Only, Osteoporosis Only, and Osteosarcopenia Groups

Study characteristics	Osteoporosis only	Sarcopenia only	Osteosarcopenia
No. of participants	278	47	195
Age (years), mean ± SD	75.15 (9.7)	76.73 (7.2)	77.88 (8.8)
Female,n(%)	224 (80.6)[a]	13 (27.7)	75 (38.5)[c]
Educational level, n (%)			
Primary	32 (11.8)	3 (6.4)	21 (10.9)
Intermediate	204 (75.0)	35 (74.5)	135 (69.9)
High	36 (13.2)	9 (19.1)	37 (19.2)
DHD index score, mean ± SD	55.5 (10.1)	55.7 (10.0)	54.1 (11.7)
Alcohol ≥20 g/d (women) or ≥30 g/d (men)	13 (6.0)	2 (5.1)	9 (6.1)
Smoking, n (%)			
Never	101 (36.3)	15 (31.9)	48 (24.7)
Former	125 (45.0)	29 (61.7)	109 (56.2)
Current	52 (18.7)a	3 (6.4)	37 (19.1)[b]
Physical activity, median (IQR)	31.8 (11.9. 76.6)	21.6 (9.0. 61.2)	19.4 (9.0. 43.8)
Height (cm), mean (SD)	161.97 (8.52)[a]	171.56 (9.45)	167.13 (9.75)[b]
BMI (kg/m^2), mean (SD)	26.40 (4.23)[a]	24.26 (2.57)	23.57 (2.89)[c]
Normal (BMI 18.5–25)	115 (41.4)	31 (66.0)	142 (72.8)
Overweight (BMI 25–30)	114 (41.0)	15 (31.9)	48 (24.6)
Obese (BMI 30+)	49 (17.6)	1 (2.1)	5 (2.6)
WHR, mean (SD)	0.87 (0.10)	0.92 (0.10)	0.91 (0.11)
Femoral neck BMD (g/m^2), mean ± SD	0.66 (0.05)[a]	1.04 (0.09)	0.78 (0.11)[b, c]
Disability index, n (%)			
No disability	158 (67.2)	20 (51.3)	99 (58.2)
Mild disability	54 (23.0)	13 (33.3)	50 (29.4)
Severe disability	23 (9.8)	6 (15.4)	21 (12.4)
MMSE, n (%)			
Cognitive impairment (<26)	40 (14.4)	7 (14.9)	32 (16.4)
No cognitive impairment (≥26)	238 (85.6)	40 (85.1)	163 (83.6)
Living status, n (%)			
Independent	225 (80.9)	44 (93.6)	163 (84.0)
Other	53 (19.1)	3 (6.4)	31 (16.0)
Hospitalization, yes,n(%)	24 (16.9)	7 (25.0)	22 (19.0)
Pain, yes, n(%)	125 (76.7)	21 (65.6)	80 (60.2)
Falls, yes,n(%)	50 (30.7)	13 (40.6)	34 (25.6)

DHD = Dutch health diet; BMI = body mass index; WHR = waist hip ratio; MMSE = Mini-Mental State Examination.

p value <0.05, adjusted for age, sex, and cohort effect:

a sarcopenia only versus osteoporosis only;

b sarcopenia only versus osteosarcopenia;

c osteoporosis only versus osteosarcopenia. For the group comparisons analyses, physical activity was log transformed.

Table 5 | Comparing Clinical Characteristics Between Sarcopenia Only, Osteoporosis Only, and Osteosarcopenia Groups

Study characteristics	Osteoporosis only	Sarcopenia only	Osteosarcopenia
	278	47	195
History of nonvertebral fracture,n(%)	96 (34.5)a	8 (17.0)	47 (24.1)
Metabolic syndrome,n(%)	64 (24.2)	11 (24.4)	26 (13.9)[c]
Blood pressure >130/85 mmHg	213 (76.6)	37 (80.4)	157 (80.9)
Coronary heart disease,n(%)	6 (2.2)	4 (8.7)	14 (7.3)
Diabetes mellitus,n(%)	20 (7.4)	9 (19.6)	15 (7.9)[b]
COPD,n(%)	57 (20.5)	11 (23.4)	56 (28.7)
Stroke,n(%)	21 (7.6)	6 (12.8)	12 (6.2)
Neoplasm,n(%)	19 (13.4)	6 (21.4)	21 (18.3)
Depression,n(%)	32 (12.9)	3 (7.0)	16 (9.1)

COPD = chronic obstructive pulmonary disease.
p value <0.05, adjusted for age, sex, and cohort effect:
a sarcopenia only versus osteoporosis only;
b sarcopenia only versus osteosarcopenia;
c osteoporosis only versus osteosarcopenia.

Table 6 | Comparing Biochemical Characteristics Between Sarcopenia Only, Osteoporosis Only, and Osteosarcopenia Groups

Study characteristics	Osteoporosis only	Sarcopenia only	Osteosarcopenia
Cholesterol (mmol/L), mean ± SD	5.5 ± 1.05	4.9 ± 1.09	5.2 ± 1.07
HDL cholesterol (mmol/L), mean ± SD	1.6 ± 0.46	1.37 ± 0.42	1.5 ± 0.43
Triglycerides (mmol/L), mean ± SD	1.3 ± 0.55	1.4 ± 0.75	1.3 ± 0.61
Aspartate transaminase (U/L), median (IQR)	24.0 (21.0–27.0)	24.0 (21.0–26.0)	24.0 (22.00–28.0)
ALT (U/L), median (IQR)	16.0 (13.0–21.0)	15.5 (13.0–21.8)	18.0 (14.00–22.0)
ALP (U/L), median (IQR)	72.0 (61.0–84.0)	63.5 (53.3–80.0)	70.0 (61.00–87.0)[b]
Bilirubin (μmol/L), median (IQR)	8.0 (6.0–10.0)	9.00(7.0–13.0)	8.0 (6.00–10.0)[b, c]
GGT (U/L), median (IQR)	21.0 (15.5–30.5)	23.5 (15.5–32.5)	23.0 (17.00–39.5)
Creatinine (μmol/L), median (IQR)	74.0 (65.0–85.0)	86.5 (71.0–108.0)	80.0 (68.00–97.0)
Hemoglobulin (g/dL), mean ± SD	8.4 ± 0.7[a]	8.5 ± 0.9	8.6 ± 0.8
Glucose (mmol/L), median (IQR)	5.3 (5.0–5.8)	5.4 (5.0–6.3)	5.4 (5.10–6.1)
Insulin (pmol/L), median (IQR)	64.0 (43.5–88.0)	61.0 (43.3–78.5)	64.0 (45.00–92.0)
HOMA-IR, median (IQR)	2.2 (1.4–3.3)	2.0 (1.6–3.6)	2.3 (1.46–3.3)

HDL = high-density lipoprotein; ALT = alanine aminotransferase; ALP = alkaline phosphatase; GGT = gamma-glutamyl transferase; HOMA-IR = homeostatic model assessment-insulin resistance.
p value <0.05, adjusted for age, sex, and cohort effect:
a sarcopenia only versus osteoporosis only;
b sarcopenia only versus osteosarcopenia;
c osteoporosis only versus osteosarcopenia. For the group comparison analyses, the variables presented as median (IQR) were log transformed.

group had higher prevalence of diabetes mellitus (**Table 5**) and higher levels of bilirubin and lower levels of ALP (**Table 6**).

In total 80.4% of the individuals with sarcopenia had low bone mineral density (T-score <–1 SD) (**Figure 3A**). However only 15% of the individuals with osteoporosis were sarcopenic (**Figure 3B**). Being presarcopenic or sarcopenic did not increase the risk of fracture. Individuals with osteoporosis and no (pre) sarcopenia had similar fracture risk (OR: 2.75, 95% CI 2.01-3.75) compared with individuals with osteoporosis and presarcopenia (OR: 3.63, 95% CI 1.83-7.21) or osteoporosis and sarcopenia (OR = 2.59, 95% CI 1.41-4.75) using the individuals with BMD and lean mass in the normal

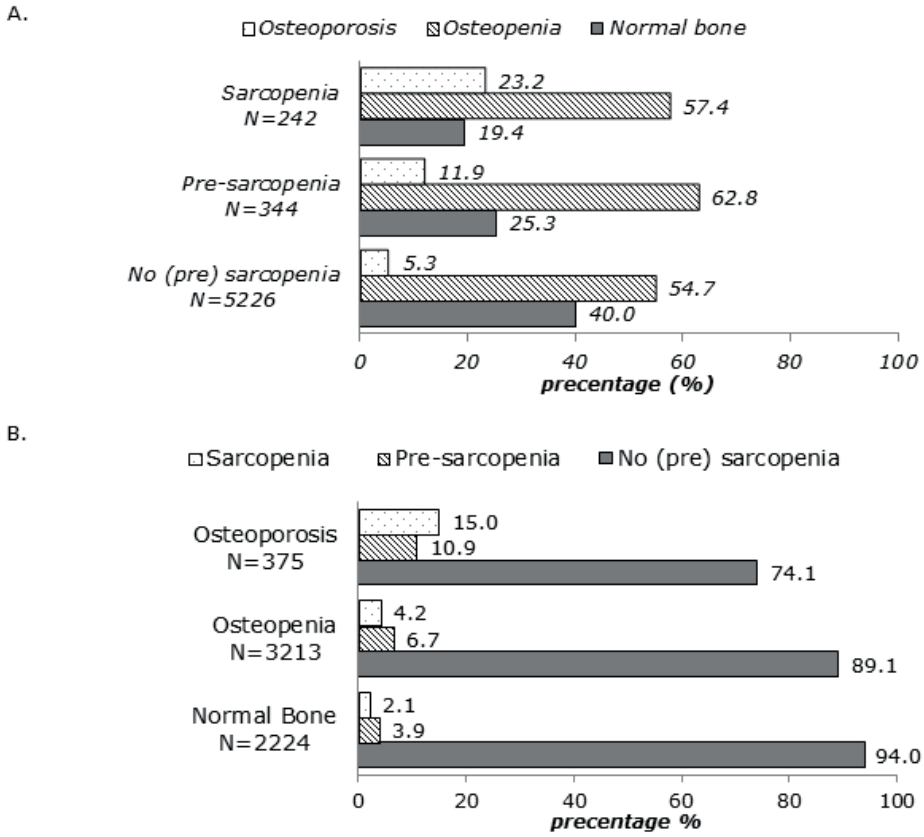


Figure 3 | Bone mineral density status according to the no (pre) sarcopenia, presarcopenia, and sarcopenia groups (A). Sarcopenia status according to the osteoporosis, osteopenia, and normal bone groups (B).

range as reference (**Figure 4**). Furthermore, sarcopenia had no effect even after using T-score in continuous manner (**Supplemental Table 1**).

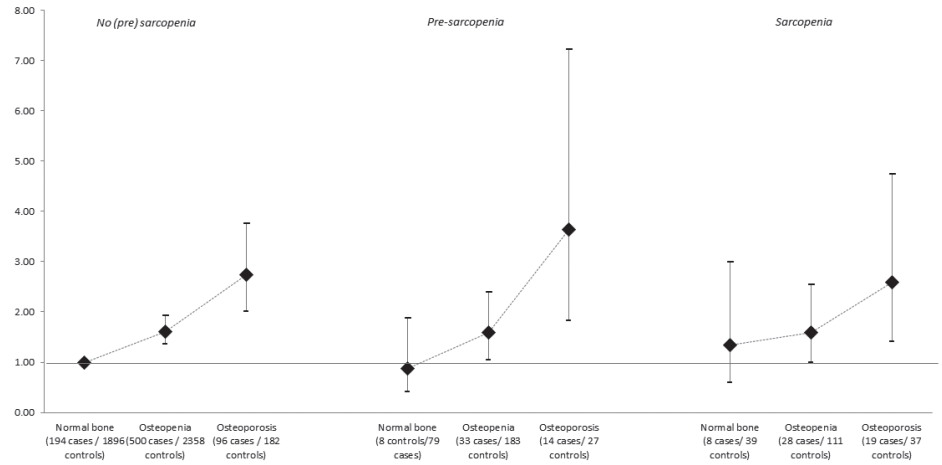


Figure 4 | Fracture risk per different bone mineral density status and sarcopenia status groups compared with individuals with normal bone and no (pre)sarcopenia

DISCUSSION

In this large population-based cohort (n = 5,911), the prevalence of sarcopenia according to the EWGSOP definition was 4.4% (95% CI 3.9-5.0). The prevalence of sarcopenia increased progressively with advancing age and was higher in men than in women. Individuals with sarcopenia were more likely to be smokers and were less physically active. Comorbid conditions were more frequently observed in the sarcopenia group; yet, with the exception of COPD, this finding was mainly explained by age and sex. In total, 80.6% of the sarcopenic individuals had a low bone mineral density. Individuals with osteoporosis, regardless of their sarcopenic status, had greater risk of fracture compared with individuals with normal BMD and normal lean mass.

Previous studies reported substantial differences in prevalence estimates of sarcopenia, fluctuating between 0.9% and 7.5% applying the EWGSOP criteria across population-based studies. Even higher prevalence's of up to 33% were observed in some patient-based settings.[29] These differences in prevalence can be attributed to several reasons, including variation in the instruments used to assess the sarcopenia components; the employed reference population; applied definition and cut-off points; the age range of the included individuals; and regional/ geographical variations. For example, a relatively low prevalence (0.9%) was reported by Patil and colleagues [2] among Finnish community-dwelling women aged 70 to 80 years. This is likely the result of selection bias because women suffering from diverse degenerative conditions were excluded, leaving mostly healthy women in the study. On the contrary, a relatively high prevalence was reported by Volpato and colleagues [4] (7.5%; 95%CI

0.06-0.10) and Patel and colleagues[5] (6.8%; 95%CI 0.03-0.13) among population-based individuals aged 70 to 74 years. However, the CIs of the prevalence's from the previous studies were quite close or overlapped with our Cis, meaning that these differences are not significantly different. However, the higher prevalence in the study by Volpato and colleagues can be caused by the high age of the population and the use of bioelectrical impedance analysis (BIA), which can over/underestimate the lean mass.[30] The equations used to estimate lean mass from BIA differ, leading to varying lean mass estimates depending on the equation used. A prevalence similar to our observation was reported by Kemmler and colleagues[31] (4.6%; 95% CI 0.03–0.06 only in women) and Spira and colleagues[32] (4.1%; 95% CI 0.03-0.05). The latter study population was similar to our population; however, the study population by Kemmler and colleagues was older than ours and used different cut-off points for the sarcopenia components.

Presarcopenic and sarcopenic individuals were more likely to be current smokers (presarcopenia 23.7%; sarcopenia 16.6%; nonsarcopenia 14.6%) and the difference remained significant after adjustment for age and sex. Although several epidemiological studies have shown association between cigarette smoking and increased prevalence of sarcopenia in elderly longtime smokers,[33] a recent meta-analysis showed that the results across studies are largely inconsistent.[34] Previous studies have also reported an inverse association between education level and sarcopenia,[4] but we did not find differences between educational levels. Adequate nutrition and regular physical exercise are the cornerstones of the prevention and treatment of sarcopenia and osteoporosis.[35-37] In line with this, in our study individuals in the sarcopenia group indeed were less physically active, reinforcing its role as an important lifestyle factor in the onset and prevention of sarcopenia. The differences in diet quality in our study were mainly driven by age and sex.

Multiple factors and processes can induce muscle dysfunction leading to the development of sarcopenia. The most prominent ones include physical inactivity, oxidative stress (neuronal degeneration, reduced force generation) and inflammation (elevated interleukin-6 and tumor necrosis factor-alpha). Many diseases can cause muscle dysfunction leading to the development of sarcopenia operating via these mechanisms. With the exception of COPD, we found no significant evidence of sarcopenia being affected or determined by other comorbidities. COPD is a condition presenting with muscle dysfunction and low functional performance. [38] In our study, COPD was more frequently observed in individuals with presarcopenia and sarcopenia, which (unlike other conditions) remained significantly associated after correction for confounding of age and sex. These differences also remained significant after controlling for smoking, physical activity, and number of prescribed corticosteroids (data not shown). These results should be interpreted with caution given that the differences could be affected by other unaccounted confounders. Currently, there

is no clear mechanism underlying the relationship between COPD and sarcopenia. Moreover, a comprehensive summary of the current literature can help us depict the nature of the relationship between COPD and sarcopenia.

Other associations with metabolic factors were significant but not in line with biological expectations, like individuals with sarcopenia holding better lipid profile and less prevalence of metabolic syndrome. These findings make unlikely that other disease processes (with the exception of COPD) are triggered together or by sarcopenia unless supported otherwise by new evidence arising from future well-powered studies. Noteworthy, individuals in the sarcopenia group had a higher prevalence of fractures (sarcopenia 23.5%; nonsarcopenia 15.5%) and presented on average a 0.05 to 0.10 g/cm^2 lower BMD. When we compared fracture risk in sarcopenic and presarcopenic individuals who were in the normal BMD range (T-score >-1SD) with those with osteopenia (T-score >-2.5 and <-1), we identified a trend toward higher risk of fracture in the pre- and sarcopenic groups. The relationship between lean mass and BMD is well established and attributed to mechanical and biochemical interactions.[39,40] Sarcopenia often coexists with osteopenia and/or osteoporosis, and this group of individuals has the highest risk of developing future fractures. [41] Low BMD is associated with fractures, which in turn leads to immobilization and disuse of the lower limbs, resulting in reduced bone and muscle mass. However, we could determine that confounding by age and sex in our study mainly drove the differences in fracture prevalence and fracture risk. In line with a previous study,[42] we showed that sarcopenia does not increase the risk of fracture. Furthermore, 74% of the individuals with osteoporosis did not have low lean mass (11% were presarcopenic and 15% sarcopenic). Interestingly enough, the majority of sarcopenic individuals had low bone mineral density (T-score <-1SD). One theory to explain the latter phenomenon is that low muscle mass cannot exert proper mechanical stimuli on the bone, thus leading to decline in BMD. [43] Yet, the presence of osteosarcopenia is unlikely to exert a higher liability than what low BMD alone exerts on fracture risk. Our study has several strengths as it is performed in a large prospective population-based setting offering the advantage of being able to use standardized methods to define and assess sarcopenia at the population level. Yet, our study is not free of limitations. First, while awaiting follow-up measurements, our study employs a cross-sectional design, which restricts the ability to infer causal relationships between sarcopenia and its clinical correlates. A cross-sectional design can be prone to reverse causation. For example, in our study, we cannot determine if individuals are more disabled because of sarcopenia or if disability leads to sarcopenia. Furthermore, our study setting comprises a follow-up evaluation of the population, which can make it liable to survival bias. Similarly, the measurement of walking speed may present another form of selection bias, as individuals with severe comorbid conditions are not able to walk and be assessed. Therefore, it is possible that

the prevalence of sarcopenia in our study is underestimated. Furthermore, despite our attempts of being as thorough as possible in data acquisition, there was a relatively high percentage of missing data on important covariates like falls and hospitalization. Perhaps this may explain why we do not see significant differences between groups. The dietary intake for RSIII-2 was accessed in the previous visit (RSIII-1), although it has been shown that diet patterns remain relatively constant over time.[44] Yet, there is the possibility that this can have an impact on the comparisons between the groups. Furthermore, in our study, handgrip was measured on the nondominant hand and the cut-off created were based on the dominant hand, which can underestimate the prevalence of low grip strength. In addition, a complete assessment of sarcopenia was only possible during the latest follow-up assessment where total body lean mass and muscle strength/function measurements were both available.

In conclusion, in this population-based cohort, the prevalence of sarcopenia is relatively low but shown to increase markedly with age, where 1 in 5 men and 1 in 10 women are sarcopenic by the age of 80 years. Lifestyle factors seem to play an important role in the prevalence of sarcopenia. COPD was the only clinical risk factor that was more prevalent in individuals with presarcopenia and sarcopenia. Overall, our findings suggest that sarcopenia does not add additional risk for fracture. The majority of individuals with sarcopenia have low bone mineral density, suggesting that co-occurrence of both conditions may follow similar pathways and be responsive to similar interventions.

Detailed acknowledgments and online resources can be found in the published article online

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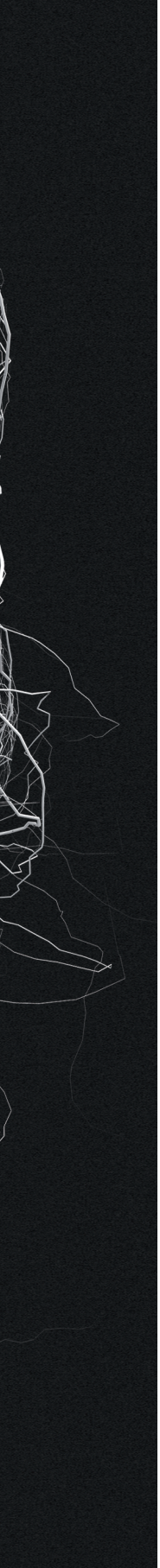
REFERENCES

1. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al. Sarcopenia: Europeanconsensus on definition and diagnosis: report of the EuropeanWorking Group on Sarcopenia in Older People. Age Ageing.2010;39:412–23.
2. Patil R, Uusi-Rasi K, Pasanen M, Kannus P, Karinkanta S, Siev€anen H.Sarcopenia and osteopenia among 70–80-year-old home-dwellingFinnish women: prevalence and association with functionalperformance. Osteoporos Int. 2013;24:787–96.
3. Legrand D, Vaes B, Mathe€ıC, Swine C, Degryse J-M. The prevalence ofsarcopenia in very old individuals according to the Europeanconsensus definition: insights from the BELFRAIL study. Age Ageing.2013;42:727–34.
4. Volpato S, Bianchi L, Cherubini A, et al. Prevalence and clinicalcorrelates of sarcopenia in community-dwelling older people:application of the EWGSOP definition and diagnostic algorithm.J Gerontol A Biol Sci Med Sci. 2014;69:438–46.
5. Patel HP, Syddall HE, Jameson K, et al. Prevalence of sarcopenia incommunity-dwelling older people in the UK using the EuropeanWorking Group on Sarcopenia in Older People (EWGSOP) definition:findings from the Hertfordshire Cohort Study (HCS). Age Ageing.2013;42:378–84.
6. Bastiaanse LP, Hilgenkamp TIM, Echteld MA, Evenhuis HM.Prevalence and associated factors of sarcopenia in older adultswith intellectual disabilities. Res Dev Disabil. 2012;33:2004–12.
7. Rizzoli R, Reginster J-Y, Arnal J-F, et al. Quality of life in sarcopeniaand frailty. Calcif Tissue Int. 2013;93:101–20.
8. Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass(sarcopenia)inolderpersonsisassociatedwithfunctionalimpairmentand physical disability. J Am Geriatr Soc. 2002; 50:889–96.
9. Beaudart C, Rizzoli R, Bruyere O, Reginster J-Y, Biver E. Sarcopenia:burden and challenges for public health. Arch Public Health. 2014;72:45.
10. Cesari M, Landi F, Vellas B, Bernabei R, Marzetti E. Sarcopenia andphysical frailty: two sides of the same coin. Front Aging Neurosci.2014;6:192.
11. Cederholm T. Overlaps between frailty and sarcopenia definitions.Nestle Nutr Inst Workshop Ser. 2015;83:65–9.
12. Chalhoub D, Cawthon PM, Ensrud KE, et al. Risk of nonspine fracturesin older adults with sarcopenia, low bone mass, or both. J Am GeriatrSoc. 2015;63:1733–40.
13. Hofman A, Brusselle GGO, Darwish Murad S, et al. The RotterdamStudy: 2016 objectives and design update. Eur J Epidemiol. 2015;30:661–708.
14. Delmonico MJ, Harris TB, Lee J-S, et al. Alternative definitions ofsarcopenia, lower extremity performance, and functional im-pairment with aging in older men and women. J Am Geriatr Soc.2007;55:769–74.
15. Fried LP, Tangen CM, Walston J, et al. Frailty in older adults: evidencefor a phenotype. J Gerontol A Biol Sci Med Sci. 2001;56:M146–57.
16. GAITRite Systems. The one and only [Internet]. Available at: http://www.gaitrite.com. Accessed June 19, 2017.
17. Verlinden VJA, van der Geest JN, Hofman A, Ikram MA. Cognition andgait show a distinct pattern of association in the general population.Alzheimers Dement. 2014;10:328–35.

18. Looker AC, Wahner HW, Dunn WL, et al. Updated data on proximalfemur bone mineral levels of US adults. Osteoporos Int. 1998;8:468–89.
19. Jones-McLean EM, Shatenstein B, Whiting SJ, et al. Dietary patternsresearch and its applications to nutrition policy for the prevention ofchronic disease among diverse North American populations. ApplPhysiol Nutr Metab. 2010;35:195–8.
20. Stel VS, Smit JH, Pluijm SM, Visser M, Deeg DJ, Lips P. Comparison ofthe LASA Physical Activity Questionnaire with a 7-day diary andpedometer. J Clin Epidemiol. 2004;57:252–8.
21. Caspersen CJ, Bloemberg BP, Saris WH, Merritt RK, Kromhout D. Theprevalence of selected physical activities and their relation withcoronary heart disease risk factors in elderly men: the ZutphenStudy, 1985. Am J Epidemiol. 1991;133:1078–92.
22. Folstein MF, Folstein SE, McHugh PR."Mini-mental state."A practicalmethod for grading the cognitive state of patients for the clinician.J Psychiatr Res. 1975;12:189–98.
23. Fries JF, Spitz PW, Young DY. The dimensions of health outcomes:the health assessment questionnaire, disability and pain scales.J Rheumatol. 1982;9:789–93.
24. Leening MJG, Kavousi M, Heeringa J, et al. Methods of data collectionand definitions of cardiac outcomes in the Rotterdam Study. Eur JEpidemiol. 2012;27:173–85.
25. Ligthart S, van Herpt TTW, Leening MJG, et al. Lifetime risk ofdeveloping impaired glucose metabolism and eventual progressionfrom prediabetes to type 2 diabetes: a prospective cohort study.Lancet Diabetes Endocrinol. 2016;4:44–51.
26. van Durme YMTA, Verhamme KMC, Stijnen T, et al. Prevalence,incidence, and lifetime risk for the development of COPD in theelderly. Chest. 2009;135:368–77.
27. Bos MJ, Koudstaal PJ, Hofman A, Ikram MA, Koudstaal P. Modifiableetiological factors and the burden of stroke from the RotterdamStudy: a population-based cohort study. PLoS Med. 2014;11:e1001634.
28. Radloff LS. The CES-D Scale: a self-report depression scale forresearch in the general population. Appl Psychol Meas. 1977;1:385–401.
29. Landi F, Liperoti R, Fusco D, et al. Prevalence and risk factors ofsarcopenia among nursing home older residents. J Gerontol A BiolSci Med Sci. 2012;67A:48–55.
30. Janssen I, Heymsfield SB, Baumgartner RN, Ross R. Estimation ofskeletal muscle mass by bioelectrical impedance analysis. J ApplPhysiol. 2000;89:465–71.
31. Kemmler W, Teschler M, Goisser S,et al. Prevalence of sarcopeniain Germany and the corresponding effect of osteoarthritisin females 70 years and older living in the community: resultsof the FORMoSA study. Clin Interv Aging. 2015;10:1565.
32. Spira D, Norman K, Nikolov J, Demuth I, Steinhagen-Thiessen E,Eckardt R. Prevalence and definition of sarcopenia in communitydwelling older people. Z Gerontol Geriatr. 2016;49:94–9.
33. SteffIM, Bohannon RW, Petr M, Kohlikova E, Holmerova I. Relationbetween cigarette smoking and sarcopenia: meta-analysis. PhysiolRes. 2015;64:419–26.
34. Furberg H, Kim Y, Dackor J, et al. Genome-wide meta-analysesidentify multiple loci associated with smoking behavior. Nat Genet.2010;42:441–7.
35. Burton LA, Sumukadas D. Optimal management of sarcopenia. ClinInterv Aging. 2010;5:217–28.
36. Anderson JJ, Rondano P, Holmes A. Roles of diet and physical activityin the prevention of osteoporosis. Scand J Rheumatol Suppl.1996;103:65–74.
37. Borer KT. Physical activity in the prevention and amelioration ofosteoporosis in women: interaction of mechanical, hormonal anddietary factors. Sports Med. 2005;35:779–830.

38. Jones SE, Maddocks M, Kon SSC, et al. Sarcopenia in COPD:prevalence, clinical correlates and response to pulmonary rehabili-tation. Thorax. 2015;70:213–8.
39. Kaji H. Interaction between muscle and bone. J Bone Metab.2014;21:29–40.
40. Brotto M, Bonewald L. Bone and muscle: interactions beyondmechanical. Bone. 2015;80:109–14.
41. Ji H-M, Han J, Won Y-Y. Sarcopenia and osteoporosis. Hip Pelvis.2015;27:72–6.
42. Harris R, Chang Y, Beavers K, et al. Risk of fracture in women withsarcopenia, low bone mass, or both. J Am Geriatr Soc. 2017;65:2673–8.
43. Verschueren S, Gielen E, O'Neill TW, et al. Sarcopenia and itsrelationship with bone mineral density in middle-aged and elderlyEuropean men. Osteoporos Int. 2013;24:87–98.
44. Schoufour JD, de Jonge EAL, Kiefte-de Jong JC, et al. Socio-economicindicators and diet quality in an older population. Maturitas.2018;107:71–7.





3.3

Sarcopenia in COPD: a systematic review and meta-analysis

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ABSTRACT

COPD is associated with a progressive loss of muscle mass and function. However, there is an unmet need to define and standardise methods to estimate the prevalence of sarcopenia in COPD patients. We performed a systematic review and meta-analysis of the prevalence of this extrapulmonary manifestation in COPD patients. We searched Embase, Medline (Ovid), CINAHL (EBSCO), Web of Science, Scopus and Google Scholar for studies published up to January 17, 2019, assessing sarcopenia in COPD patients based on low muscle mass and decreased muscle function. Interventional studies, in vitro experiments, protocols or reviews and meta-analyses were excluded. We estimated heterogeneity (I^2) and assessed significance (Q) using a Chi-squared test for estimates obtained from random-effects models. 4,465 articles were initially identified. After removing the duplicates and applying the selection criteria, we reviewed 62 full-text articles. Finally, 10 articles (n=2565 COPD patients) were included in this systematic review and meta-analyses. Overall, the prevalence of sarcopenia in patients with COPD was 21.6% (95%CI 14.6–30.9%, I^2 =94%), ranging from 8% in population-based to 21% in clinic-based studies, and 63% in COPD patients residing in nursing homes. Sarcopenia is frequently observed in COPD patients, with varying prevalence across population settings. Sarcopenia in COPD should be assessed using standardised tests and cut-off points from sarcopenia consensus criteria for clinical practice and international comparisons.

INTRODUCTION

COPD is a heterogeneous, complex and multisystem disease, with a vast number of intra- and extrapulmonary manifestations.[1-5] The clinical assessment of COPD patients is also complicated by the presence of multi-morbid conditions due to shared risk factors with other diseases[3,6]. The extrapulmonary manifestations are increasingly recognised as important contributors to functional decline in patients with COPD.[2,3] Functional impairment is associated with muscle weakness and body weight loss in COPD patients.[5-7] Irrespective of the method used to assess muscle mass,[8] it is well known that patients with COPD suffer from loss of muscle mass.[9-29] Indeed, loss of muscle mass and muscle strength can be greater in patients with moderate-to severe COPD [7,30] or during acute COPD exacerbations,[31] especially loss of muscle from the lower limbs .[6,7,32] Low muscle mass and loss of muscle function have been described as the main components of sarcopenia,[33–38] which was originally defined as a multifactorial geriatric syndrome.[39] However, there has been increasing recognition of this muscle disorder as a disease-related rather than just age-related condition. [40] The 2018 European Working Group of Sarcopenia in Older People (EWGSOP2) have reinforced the importance of distinguishing "secondary sarcopenia" when factors other than ageing are involved from "primary sarcopenia" or age-related sarcopenia.[38] Sarcopenia can result from underlying diseases such as COPD or other conditions characterised by chronic inflammation. [38] Although COPD occurs mainly in middle-aged and older people, sarcopenia in COPD can occur independent of age. [41] Therefore, early detection of sarcopenia might be pivotal, given that it has therapeutic implications such as pulmonary rehabilitation and management of respiratory failure in COPD patients. Although many aspects of the pathophysiology of sarcopenia are better understood now than a decade ago, [38] the precise aetiology of sarcopenia and underlying explanatory mechanisms are still not fully established. [42,43] However, physical inactivity, weight loss, inflammatory cytokines, inadequate energy intake, oxidative stress and reduced blood flow to muscles are common features linked with sarcopenia [43] as well as COPD. [3,5,44] For instance, it is well known that limb muscle dysfunction in COPD contributes to loss of muscle strength.[7] Despite the heterogeneity in the muscle fibre proportions in COPD patients, [45] there is a shift from type I to II fibres, which correlates inversely with disease severity.[7] By contrast, age-related loss of muscle strength and mass are mainly attributed to muscle fibre denervation and loss of type II fibres. [46] In the last decade, different international consensus criteria of sarcopenia [33-38] have focused clinically and operationally on the measurements and thresholds of sarcopenia (**Table 1**). For instance, five out of six sarcopenia consensus statements recommend muscle mass measurement as a first criterion to define sarcopenia. Only the updated EWGSOP2 recommends including muscle strength as the main criterion of sarcopenia,

and labelling the patient as having "probable sarcopenia" [38] when muscle strength is compromised. Using these consensus criteria, it has recently been reported that 10% of healthy older adults have sarcopenia. [47] However, the prevalence of sarcopenia varies widely (0.9%–32.8%) among different study populations. [48] No global estimates of the prevalence of sarcopenia in COPD patients with the consensus definitions have previously been reported. Estimating the overall prevalence of sarcopenia, according to the contemporary operational definitions recommended by multidisciplinary consensus, will provide further insight into its burden in COPD. Therefore, we conducted a systematic review and meta-analysis to appraise the best available evidence on the prevalence of sarcopenia among COPD patients based on the consensus definitions.

METHODOLOGY

Search strategy and data sources

This systematic review and meta-analysis adhere to the schema of Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA). [49,50] We performed an electronic search of Embase.com, Medline (Ovid), CINAHL (EBSCO), Web of Science, Scopus and Google Scholar to identify all relevant articles relating to sarcopenia and COPD. We searched all records published from inception through to January 17, 2019, in collaboration with a medical information specialist (GdJ). Terms related to COPD (e.g. "chronic obstructive pulmonary disease") and key terms were combined with synonyms of sarcopenia (e.g. "muscle atrophy"). We further hand-searched bibliographies of relevant publications to identify potential articles missed by the principal search. Full search terms are listed in **Supplementary Table 1**. This systematic review is registered in the International Prospective Register of Systematic Reviews (PROSPERO), number CDR42018095480.

Selection Criteria

For this systematic review, we used the following inclusion criteria: 1) all studies had to be case–control, longitudinal or cross-sectional designs; 2) assessment of sarcopenia had to be implemented according to the sarcopenia consensus criteria (muscle mass and muscle strength or physical performance are combined); 3) measurement of lean mass or muscle mass had to be assessed by at least one of four main methods [8] (bioelectrical impedance analysis, dual-energy X-ray absorptiometry, magnetic resonance imaging or computed tomography); 4) the prevalence of sarcopenia (primary outcome) in COPD patients had to be reported. To provide additional insight into the prevalence of sarcopenia in COPD patients, we also investigated the associations of sarcopenia (if available in the study, e.g. odds ratio) and COPD as a secondary outcome

measure. We limited our inclusion to original publications, without language restriction, and excluded experimental studies, reviews and meta-analyses, comments, guidelines, editorials or letters and case reports. Furthermore, studies that referred to other populations were excluded (i.e. asthma or cystic fibrosis) (supplementary table S2). Two independent co-authors (EB and KT) screened all the articles by title and abstract. Disagreements were resolved by consensus between both co-authors together with a third co-author (LL). The citations were classified as "eligible", "uncertain about eligibility" or "excluded" and stored in an EndNote file. The full text of articles classed as "eligible" and "uncertain about eligibility" was reviewed and appraised independently by two co-authors (EB and KT), and disagreements were solved through revision and consultation with a fourth independent co-author (JDS).

Data Synthesis

We used a predefined data collection form in which we summarised relevant information on characteristics of the study population (age, sex, setting), outcome assessment (prevalence of sarcopenia, definition of sarcopenia using any of the consensus criteria, cut-off points, and methods to measure handgrip and mass muscle) and the effect estimate of association (if available in the study; odds ratio adjusted for most confounders) with the corresponding precision estimate (95% confidence interval). When relevant data were not available, the corresponding authors of the selected publications were contacted to complement the electronic searches.

Risk of Bias Assessment

We assessed the quality of the selected studies using an adapted version of the scale developed by HOY et al. [51] for prevalence estimates. The tool consists of 10 items addressing external and internal validity. Moreover, we used an adapted Newcastle–Ottawa Scale[52] for cross-sectional studies that measured association between prevalence of sarcopenia and COPD. Two co-authors (EB and KT) independently rated each of the items of both scales into a global score adding all the items rated as low risk. Therefore, higher scores indicated lower risk of bias (**Supplementary Table 3**).

STATISTICAL ANALYSIS

We summarised prevalence of sarcopenia across studies as its estimator (prevalence) and confidence intervals and present data in tables of study characteristics. Meta-analysis on the prevalence of sarcopenia in COPD patients was undertaken using a random-effects model, using the packages meta and metaprop in R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Heterogeneity across studies

was defined by an I2 of >50%.[53] Given that the preliminary analyses demonstrated high heterogeneity, we stratified our analysis by population setting to explore potential sources of heterogeneity. We used a funnel plot adjusted for population setting to also measure publication bias.[54]

RESULTS

Search Results

Our search yielded 4465 records, of which 2315 duplicates were removed, resulting in 2150 unique articles. We read the full text of 62 articles, and determined that 10 of them were eligible for inclusion in this meta-analysis (**Figure 1**). [55-64]

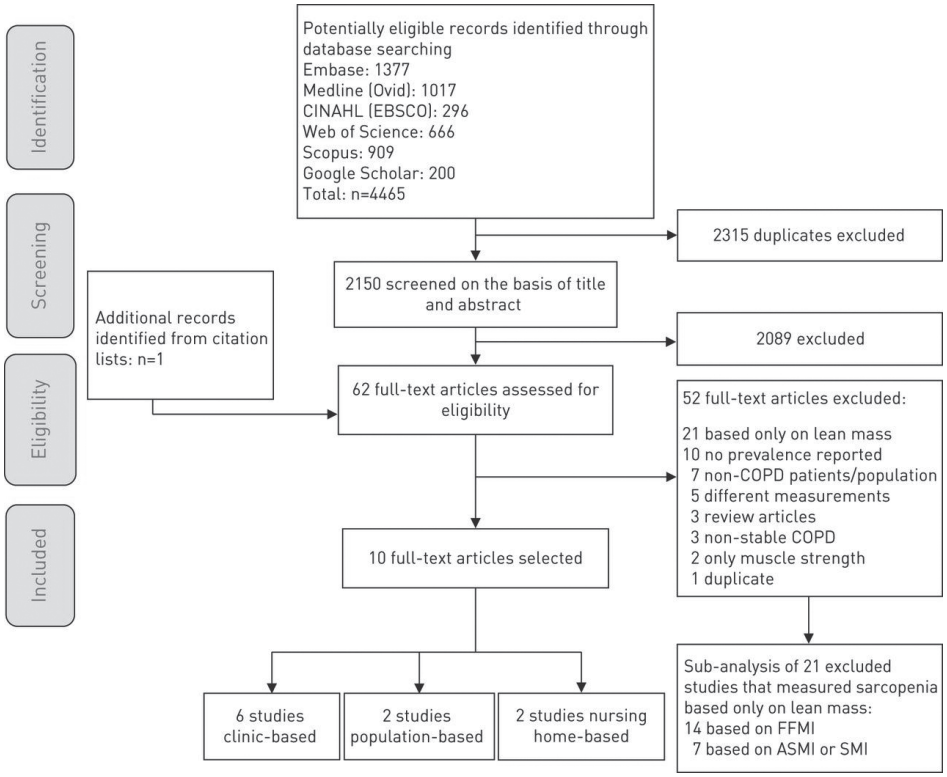


Figure 1 | Flow diagram of the study selection process. FFMI: fat-free mass index; ASMI: appendicular skeletal muscle mass index; SMI: skeletal muscle mass index.

Characteristics of Studies Included

Among the 10 eligible studies published between 2012 and 2018, six were performed only in COPD patients without a control group,[55-57,59,60,63] two reported data on population-based prevalence [58,61] and two were nursing home based[62,64]. Four studies were from Europe, [55,58,63,64] four from Asia[57,59,60,62] and two from South America.[56,61] The total number of COPD participants involved in all selected studies was 2565, with a mean age ranging from 62 to 84 years at study entry (**Table 2**). Eight of the selected studies used the EWGSOP definition of sarcopenia, [55,58-64] and two of them applied the Foundation for the National Institutes of Health (FNIH) and the Asian Working Group on Sarcopenia consensus (**Table 1**).[56-57] When we analysed the components of sarcopenia in these 10 studies, we found large variability in the methods and cut-off points, especially for lean mass. All these studies assessed lean mass by dual-energy X-ray absorptiometry or bioelectrical impedance rather than magnetic resonance imaging or computed tomography, although one used calf circumference to measure lean mass (**Table 2**).[61]

Quality Assessment

Quality assessment scores of all included observational studies are listed according to prevalence, and two of them according to association estimators (supplementary tables S4 and S5). Regarding methodological quality of the prevalence estimator, seven (70%) had moderate risk of bias and three (30%) had low risk of bias.

Prevalence of Sarcopenia in COPD Patients

The prevalence of sarcopenia in COPD patients ranged from 7.9% to 66.7%. There was substantial heterogeneity between studies (I^2=94%, p <0.01), which could largely be accounted for by two studies.[62,64] These two nursing home-based studies, which had the smallest combined sample size (n=46 COPD patients) of any study included, reported the highest prevalence of sarcopenia. When excluding these studies in a sensitivity analysis, the prevalence of sarcopenia was 16.5% (95%CI 11.4%-23.2%), with high heterogeneity (I^2 =92%, p>0.01) (**Supplementary Figure 1**). Therefore, we included the 10 studies and stratified our analysis according to the three main population settings: 1) clinic based, 2) population based and 3) nursing home based. Stratifying our analysis resulted in a reduction of heterogeneity within the three population settings. Consequently, the pooled prevalence of sarcopenia was 21.6% (95%CI 14.6%–30.9%) (**Figure 2 and Supplementary Table 6**). There was symmetry for the funnel plot describing publication bias in these 10 selected studies when stratified by population setting (**Supplementary Figure 2**). Additionally, we meta-analysed all the excluded studies that measured only the lean mass component of sarcopenia given that, prior to 2010, having a low lean mass was considered to represent sarcopenia (**Figure 1**).[34,38,40]

Table 1 | Criteria and cut-off points to diagnose sarcopenia according to the main consensus criteria groups

Consensus group	Main criteria definition	Lean muscle mass	Muscle function			
			Handgrip strength	Gait speed m·s−1	SPPB	TUG
ESPEN SIG[33]	Low muscle mass plus low gait speed	2sdbelow mean of young adults of the same sex and ethnic background		<0.8 (4MGS)		
EWGSOP[34]	Low muscle mass plus low muscle strength or low gait speed	ASMI: DXA score Male ≤7.26 kg·m^{-2} Female ≤5.54 kg·m^{-2} (2sdbelow mean of young adults) BIA score: Male 8.87 kg·m^{-2} Female 6.42 kg·m^{-2} (2sdbelow mean of young adults)	Male <30 kg Female <20 kg	<0.8 (4MGS)	≤8	
IWGS task force[35]	Low ASMI plus low gait speed	ASMI: Male ≤7.23 kg·m^{-2} Female ≤5.67 kg·m^{-2}		<1 (4MGS)		
FNIH[36]	Low ALM/BMI plus low handgrip	ALM/BMI Male <0.789 Female <0.512	Male <26 kg Female <16 kg kg/BMI Male <1 Female <0.56	<0.8 (4MGS, 6MWT)		
AWGS[37]	Low ASMI plus low gait speed or low handgrip	ASMI: Male ≤7.0 kg·m^{-2} Female ≤5.4 kg·m^{-2}	Male <26 kg Female <18 kg	≤0.8 (6MWT)		
EWGSOP2[38]	Low muscle strength plus low muscle mass; severity of sarcopenia involves low physical performance	ASMI: Male <7.0 kg·m^{-2} Female <5.5 kg·m^{-2}	Male <27 kg Female <16 kg	≤0.8	≤8	≥20 s

SPPB: Short Physical Performance Battery; TUG: timed up and go test; ESPEN SIG: European Society of Clinical Nutrition and Metabolism Special Interest Group ("cachexia-anorexia in chronic wasting diseases"); EWGSOP: European Working Group on Sarcopenia in Older People; IWGS: International Working Group on Sarcopenia; FNIH: Foundation for the National Institutes of Health; AWGS: Asian Working Group on Sarcopenia; 4MGS: 4-min gait speed; ASMI: appendicular skeletal muscle mass index; DXA: dual-energy X-ray absorptiometry; BIA: bioelectrical impedance analysis; BMI: body mass index; ALM: appendicular lean mass; 6MWT: 6-min walk test

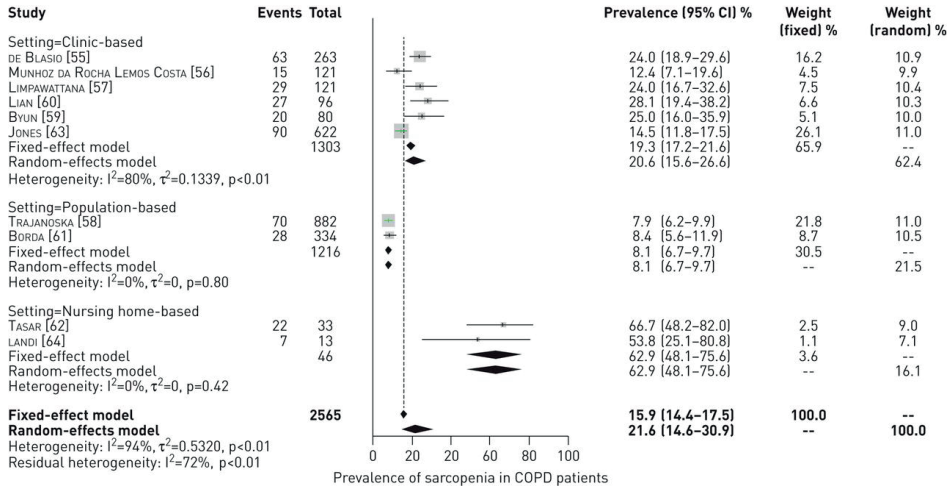


Figure 2 | Fixed- and random-effects meta-analysis results for the prevalence of sarcopenia in patients with COPD, according to population setting. The authors directly provided the prevalence estimate for the study by Tasar*et al.* [62].

Additionally, we meta-analysed all the excluded studies that measured only the lean mass component of sarcopenia given that, prior to 2010, having a low lean mass was considered to represent sarcopenia (**Figure 1**).[34,38,40] These studies applied two main muscle indices: appendicular skeletal muscle mass index (ASMI), also referred as skeletal mass index (SMI),[9-15] and fat-free mass index (FFMI).[16-29] The prevalence of low lean mass based on ASMI in COPD patients was 31.0% (95%CI 28.2%–34.2%, I^2 =65%, p<0.01). According to FFMI, the prevalence of low lean mass in COPD patients was 23.4% (95%CI 18.6%–29.0%, I^2 =96%, p<0.01) (**Supplementary Figure 3; Supplementary Tables 7, 8, 9**). After performing this division by index muscle, the I^2 statistics (65% and 96% respectively, p<0.01), although lower than when using the sarcopenia consensus criteria, still indicated heterogeneity.

Association of Sarcopenia and COPD

In addition to the prevalence of sarcopenia described in the aforementioned studies, despite the considerable variation of the cut-off points, the association of sarcopenia with COPD was tested in two studies. BORDA et al.[61] investigated the association of sarcopenia in 250 COPD/asthma participants, and the odds ratio for sarcopenia was 2.01 (95%CI 1.21–3.35), independent of sex, age, educational level and body mass index (BMI). Moreover, TASAR et al. [62] reported that the odds ratio for elderly people with COPD to have sarcopenia was 1.69 (95%CI 0.83–3.43). Factors associated with sarcopenia in COPD patients Some of the main factors associated with sarcopenia in COPD patients were age[56,59,60], especially among the older group (⩾75 years

Table 2 | Main characteristics of the cross-sectional selected studies in the meta-analysis

First author [ref.]	Country	Subjects n	Male	Age years	Setting	Consensus group	Prevalence % (n)
DeBlasio[55]	Italy	COPD: 263	70.3	69.8±8.0	Clinical	EWGSOP	24 (63)
Munhoz daRocha LemosCosta[56]	Brazil	COPD: 121	46.3	67.9±8.6	Clinical	FNIH	12.4 (15)
Limpawattanaz[57]	Thailand	COPD: 121	92.6	>65	Clinical	AWGS	24 (29)
Trajanoska[58]	Netherlands	COPD: 882		69.2±9.1	Population	EWGSOP	COPD: 7.9 (70)
		All population: 5911					All population: 4.4 (260)
Byun[59]	Korea	COPD: 80	83.8	68.4±8.9	Clinical	EWGSOP	25 (20)
Lian[60]	China	COPD: 96	58.3	62.4±7.9	Clinical	EWGSOP	28.1 (27)
Borda[61]	Colombia	COPD/asthma: 334	32.9	>60	Population	EWGSOP	COPD/asthma: 11 (28)
		No COPD/asthma: 1666					No COPD/asthma: 6.9 (77)
Tasar#[62]	Turkey	COPD: 33	69.7	73.9±7.2	Nursing home	EWGSOP	COPD: 66.7 (22)
		All population: 211					All population: 33.6 (71)
Jones[63]	UK	COPD: 622	58.8	73±8	Clinical	EWGSOP	14.5 (90)
Landi[64]	Italy	COPD: 13	68	84±7.3	Nursing home	EWGSOP	COPD: 53.4 (7)
		All population: 122					All population: 32.8 (40)

Data are presented as % or mean±SD, unless otherwise stated. EWGSOP: European Working Group Older People; M: male; F: female; BIA: bioelectrical impedance analysis; SMI: skeletal muscle index; NS: not specified; HGS: handgrip strength; 4MGS: 4-min gait speed; FNIH: Foundation for the National Institute of Health; DXA: dual-energy X-ray absorptiometry; ALM: appendicular lean mass; BMI: body mass index; 6MWT: 6-min walk test; AWGS: Asian Working Group on Sarcopenia; ASMI: appendicular skeletal muscle mass index; GAITRite: 5.79-m-long electronic walkway; CC: calf circumference; FFM: fat-free mass; BSA: body surface area. #: data directly obtained from authors.

Components of sarcopenia					
Lean mass		Muscle strength		Physical performance	
Method	Cut-off	Method	Cut-off	Method	Cut-off
BIA (SMI)	M: ≤8.50 kg·m^{-2}	HGS	NS	4MGS	<0.8
	F: ≤5.75 kg·m^{-2}				
DXA (ALM/BMI)	M: <0.789	HGS	NS	6MWT	<0.8
	F: <0.512				
DXA (ASMI)	M: ≤7.0 kg·m^{-2} F: ≤5.4 kg·m−2	HGS	M: 26 F: 18	6MWT	<0.8
DXA (SMI)	M: ≤7.25 kg·m^{-2}	HGS	M: ≤29 (if BMI ≤24); ≤30 (if BMI ≤24.1-28); ≤32 (if BMI >28)	GAITRite	M: <0.65 (if height ≤173 cm) and <0.76 (if height >173 cm)
	F: ≤5.67 kg·m^{-2}		F: ≤17 (if BMI ≤23); ≤17.3 (if BMI ≤23.1-26); ≤18 (if BMI ≤26.1–29); ⩽21 (if BMI >29)		F: <0.65 (if height ≤159 cm) or <0.76 (if height >159 cm)
BIA (SMI)	Below 2sdof mean population	HGS	M: ≤30 F: ≤20		NS
BIA (SMI)	M: ≤7.01 kg·m^{-2}	HGS	M: ≤35.04 F: ≤21.82		NS
	F: ≤5.43 kg·m^{-2}				
CC	<31 cm	HGS	NS	4MGS	
BIA (FFM/BSA)	Below the 20th percentile of mean population	HGS	M: ≤30 F: ≤20	6MWT	≤0.8
BIA (SMI)	M: ≤8.50 kg·m^{-2}	HGS	M: ≤30 F: ≤20	4MGS	≤0.8
	F: ≤5.75 kg·m^{-2}				
BIA (SMI)	M: <8.87 kg·m^{-2}	HGS	M: ≤30 F: ≤20	4MGS	≤0.8
F: <6.42 kg·m−2					

old)[57]; systemic inflammatory biomarkers (high-sensitivity tumour necrosis factor-α (hsTNF-α));[59] and BMI, either separately (both high [57] and low [59]) or as part of the BODE index (BMI, airflow obstruction, dyspnoea and exercise) [56] (supplementary table S10). Smoking, one of the main risk factors for COPD, was associated with sarcopenia in one study, [60] whereas in two other studies it was not.[57,56] In addition, JONES et al. [63] did not find a difference in smoking status between COPD patients with and without sarcopenia. Severity of COPD, [57] severity of dyspnoea [57] and cardiovascular disease[59] were the main clinical outcomes associated with sarcopenia in COPD patients. DE BLASIO et al. [55] reported that 58.7% of COPD patients with sarcopenia were also malnourished. In the study of JONES et al., [63] COPD patients with sarcopenia had more severe airflow obstruction (low forced expiratory volume in 1 s (FEV1)) and reduced physical activity, functional performance and exercise capacity. When the components of sarcopenia were analysed separately, BYUN et al. [59] found that handgrip and SMI were negatively correlated with serum interleukin-6 (r= −0.27 and r= −0.23, respectively) and hsTNF-α (r= −0.35 and r= −0.25, respectively).

Table 3 | EWGSOP2 algorithm for case finding, making a diagnosis and quantifying severity of sarcopenia in clinical practice with clinical and research considerations for COPD patients

Steps	Components	Response	Considerations for COPD patients
Step 1: Case finding	SARC-F or clinical suspicion	+: Go to step 2	SARC-F in all COPD patients
		−: No sarcopenia, rescreen later	
Step 2: Assess	Muscle strength (grip strength or chair stand test)	+: Go to step 3 plus start intervention: "probable sarcopenia"	Call for research directed to define accurate cut-off points for low muscle strength in COPD patients
		−: No sarcopenia, rescreen later	
Step 3: Confirm	Muscle quantity or quality (DXA, BIA, CT, MRI)	+: Go to step 4 plus intervention: "sarcopenia"	Call for research directed to define accurate cut-off points for low muscle mass in COPD patients
		−: Intervention for "probable sarcopenia"	
Step 4: Severity	Physical performance (gait speed, SPPB, TUG, 400 m walk)	+: "Severe sarcopenia"	Call for research directed to define optimal physical performance tests and accurate cut-off points for COPD patients

EWGSOP2: 2018 European Working Group Older People; SARC-F: questionnaire with the five components of strength, assistance with walking, rise from a chair, climb stairs and falls; DXA: dual-energy X-ray absorptiometry; BIA: bioelectrical impedance analysis; CT: computed tomography; MRI: magnetic resonance imaging; SPPB: Short Physical Performance Battery; TUG: timed up and go test.

DISCUSSION

Overall, we found that ~22% of individuals with COPD had sarcopenia. The prevalence estimates of sarcopenia varied between 12.4% and 28.1% in clinical settings, and 7.9% and 8.4% in population-based settings. Prevalence was highest in nursing home settings, ranging from 53.8% to 66.7%. We observed large heterogeneity across studies, attributed to important differences in methods and cut-off points used to assess muscle mass, and particularly the distinct population settings. Therefore, differences between study settings (i.e. community dwelling versus institutionalised patients) should be considered when assessing the risk of sarcopenia in COPD patients. In COPD patients, BONE et al.[42] have previously described the importance of sarcopenia based on consensus criteria. However, to the best our knowledge, this systematic review including a meta-analysis is the first to assess the prevalence of sarcopenia in patients with COPD. This meta-analysis notes the heterogeneity induced by the different methods used to assess sarcopenia and the type of population setting. Clinic-based studies The vast majority of the selected studies reporting prevalence of sarcopenia in COPD patients were clinic based, with high heterogeneity between studies (I^2 =80%, p<0.01). Notably, two studies using the same cut-off points and methods to measure sarcopenia in COPD patients [55,63] reported prevalence estimates with big discrepancies (~10% difference). However, the study by DE BLASIO et al. [55] (n=263) included more male COPD patients (70%), and the sarcopenic group (n=26) had more deteriorated lung function (FEV1 % pred 30.9%, 95% CI 26.1%–35.7%) than that of JONES et al. [63] (n=622) (sarcopenic group (n=90), FEV1 % pred 43.5%, 95% CI 37.4%–50.2%).

Population-based Studies

The prevalence of sarcopenia in COPD population-based studies was lower than in clinical settings. This finding can be explained by the fact that COPD cases from the population are more often in an early stage of the disease or even asymptomatic. Two population-based studies reported the prevalence of sarcopenia in COPD;[58,61] although they used the same consensus criteria, [34] there was no uniformity in the measurement methods used, particularly for lean mass. TRAJANOSKA et al. [58] performed a specific calculation of each component of sarcopenia, based on BMI and height, for male and female patients. By contrast, BORDA et al. [61] calculated low lean mass based on calf circumference for both sexes in patients with COPD/asthma. This makes comparison between the two studies difficult.

Nursing Home-based Studies

The high prevalence of sarcopenia in COPD patients from nursing homes can be explained by two main factors. First, the selected studies had small sample sizes (< 50 participants). Second, according to the mean age, these participants were older than the age range mean of the clinic- and population-based studies. However, while the prevalence of sarcopenia increases notably with ageing, the high prevalence within COPD patients is unlikely to be explained by age alone. There are biological mechanisms by which respiratory impairment contributes to muscle deterioration,[7] e.g. patients with more severe emphysema have lower muscle mass. [30]

Sarcopenia Consensus Statements

We found six international sarcopenia consensus criteria that can be applied in clinical and research fields.[33-38] All of these criteria are based on lean mass and physical performance related to muscle loss, but there is still a lack of uniformity in measurement standards, and especially in cut-off points across these criteria. However, the cut-off points for lean mass and handgrip strength measurements from two of the consensus criteria (EWGSOP2 and FNIH) are practically equivalent.[36,38] The majority of our selected studies (n=8)[55,58-64] applied the EWGSOP criteria to define sarcopenia in COPD patients. According to the updated EWGSOP2[38] recommendations, muscle strength can be considered as the principal determinant of sarcopenia, mainly because it can facilitate early identification of sarcopenia in clinical practice. EWGSOP2 have modified and simplified the cut-off points to identify primary and secondary sarcopenia in a broad population.[40] Consequently, we suggest using the EWGSOP2 practical algorithm for case finding, diagnosis of sarcopenia and determining sarcopenia severity in COPD patients. We encourage clinicians to start by systematically assessing handgrip in all COPD patients, which is easily performed, rather than with other tests that can be influenced by COPD due to ventilator restrictions. To classify the severity of sarcopenia, EWGSOP2 recommends different tests, e.g. gait speed, 400-m walk test, Short Physical Performance Battery and timed up and go test (TUG), with corresponding cut-off points. In COPD patients, an optimal test should be targeted for each case. For instance, the TUG test has not been associated with airflow limitation, in contrast to the 6-min walk distance.[65] Therefore, the TUG test could be implemented in cases of COPD with more severe ventilatory impairment. Similarly, a cut-off point (11.2s) different from the EWGSOP2 recommendation (20s) has been suggested for use as an abnormal TUG time for COPD patients. [66] Nevertheless, additional studies are needed to establish which functional test and cut-offs should be used across different stages of COPD. Given that these updated recommendations have only recently been introduced, their performance within COPD patients has not yet been performed and warrants future studies. When we analysed each element

of sarcopenia across studies, muscle mass was the component with most variability in its measurements, mainly due to muscle indices and cut-off points. For instance, in Asian populations, BYUN et al.[59] used a SMI below 2SD of the mean of the Korean population, whereas LIAN et al.[60] used cut-offs close to the ASMI. To diagnose sarcopenia in patients with COPD, dual X-ray absorptiometry and bioelectrical impedance analysis were used to measure lean mass. However, no consensus has been reached concerning cut-off points of lean mass across different international (working) groups for the diagnosis of sarcopenia. Therefore, for both SMI and ASMI, the cut-off points require further validation and standardisation. There is more consistency across the sarcopenia consensus criteria groups for assessing muscle function, although a wide range of cut-off points have been recommended (e.g. handgrip in males ranges from 26 kg to 30 kg). In addition, cut-offs for gait speed were essentially the same ($\leqslant$ or 0.8 $m \cdot s^{-1}$). Only the method to measure gait speed differed across studies: some articles used the 6-min walk test and others 4-m gait speed ($m \cdot s^{-1}$). However, 4-m gait speed is significantly correlated with the 6-min walk test in COPD patients.[67] Studies are required to further establish the role and interaction of each sarcopenia component in COPD patients.

Factors Associated with Sarcopenia in COPD Patients

We only found two studies that reported an association between sarcopenia and COPD,[58,61] even after adjustment for age, sex and BMI. Additionally, TRAJANOSKA et al.[58] reported that the difference in sarcopenia prevalence between COPD and non-COPD individuals was independent of age, sex, smoking and corticosteroid use.

Multiple factors were associated with the presence of sarcopenia in COPD patients in the selected studies. These factors included age, severity of COPD, BMI, BODE index, dyspnoea and elevated inflammatory biomarkers, such as TNF-α.[56-60] However, the direct effect of smoking on sarcopenia, an established risk factor for COPD, showed different associations across studies. For instance, only one study [60] reported an association between smoking and sarcopenia among COPD patients, whereas two other studies did not.[56,57] Likewise, in support of a negative association, another study did not find differences in smoking across COPD patients with and without sarcopenia. [63] One explanation for these discrepancies might originate from distinct smoking classification across studies; while LIAN et al.[60] used cumulative smoking exposure (i.e. a long-term history of smoking, defined as $\geqslant$10 cigarettes/day for 10 years), all other studies categorised smoking status as never, past and current.

None of these studies reported sex differences in the prevalence of sarcopenia, in contrast to what has been shown in previous studies on COPD patients that mainly focused on lean mass (and not on sarcopenia). [13,22] Moreover, COPD patients with sarcopenia had lower FEV1, lower exercise capacity and lower physical activity than

patients without sarcopenia.[63] Therefore, in terms of sarcopenia in COPD patients, factors including age, severity of COPD, BMI, BODE index, dyspnoea and hsTNF-α might add value to preventive strategies, especially when these strategies are modifiable. Likewise, other factors such as corticosteroid use, protein intake and alcohol use were not considered and as such these should be of interest for future research.

CRUZ-JENTOFT et al. [38] consider sarcopenia as "secondary" when another factor besides ageing is present. They state that secondary sarcopenia can occur due to a systemic disease (i.e. COPD), physical inactivity and inadequate energy intake, among other factors. [38] However, there is not any distinction in recommendations of methods and cut-offs for distinguishing primary and secondary sarcopenia. It is important to consider that across these selected studies, age was associated with sarcopenia, which is also in line with the fact that COPD patients with sarcopenia are older. Only one study did not find age differences in COPD patients with and without sarcopenia. [55] Nevertheless, patients in all of these studies were aged 60 years. Therefore, an overlap between primary and secondary sarcopenia in COPD patients can be expected and is difficult to differentiate. Additionally, sarcopenia is increasingly recognised as a progressive and generalised muscular disorder related to long-term conditions presenting during midlife, such as COPD. [40] Nevertheless, sarcopenia in COPD patients seems to be more disease-related than age-related, as supported by distinct physiopathological processes related to ageing and COPD. With ageing there is a transition of the muscle fibres from type II to type I, [68] involving intra- and inter-muscular adipose tissue. [69] In contrast, in COPD there is mainly a shift from type I to type II fibres[7] that can be responsible for dyspnoea symptomatology and/or low performance during exercise tests. Further understanding of the main molecular and cellular mechanisms underlying the onset of sarcopenia in COPD patients could support the optimisation of assessment tools for sarcopenia in COPD.

Strengths of this systematic review include the use of a predefined protocol, a comprehensive search strategy and the involvement of two independent co-authors in all stages of the process. Nevertheless, it is not free of limitations, and several topics should be taken into account when interpreting our results. First, as was previously highlighted, despite our results being centred around all sarcopenia consensus criteria, the cut-off points for its components were not uniform across studies. One explanation might be that there were different indices to measure muscle mass, and consequently there were different cut-offs. For instance, despite most of the selected studies used EWGSOP consensus criteria, they used different indices to measure lean mass (appendicular lean mass/height^2, skeletal muscle mass/height^2, fat-free mass/body surface area); they also applied different cut-offs even for the same muscle index. Another explanation may be that they used different dual-energy X-ray absorptiometry and bioelectrical impedance analysis devices to measure lean mass.

It is well known that bioelectrical impedance analysis, an inexpensive alternative to dual-energy X-ray absorptiometry, is dependent on the formula used, [70] which differed among these studies. It has also been widely reported that dual-energy X-ray absorptiometry and bioelectrical impedance measurements in healthy adults provide different estimates of fat mass and fat-free mass. [71] Second, the main aim of this systematic review and meta-analysis was to provide a comprehensive overview of the current overall prevalence of sarcopenia in COPD patients, independent of the population setting. However, the six clinic-based studies used different consensus criteria and were performed in different ethnic groups, resulting in different estimates of sarcopenia prevalence. Similarly, the two population-based studies used different cut-offs and measurements, as did the nursing home studies, and the quality assessment of the selected studies varied, affecting the external validity. Therefore, our results may not be representative for COPD patients in general. Likewise, our results highlight that population-based studies on the association of main lifestyle factors (e.g. diet, physical activity, smoking) with sarcopenia in COPD patients remain limited.

More studies are needed to identify the main characteristics of COPD patients who present with sarcopenia defined by low muscle mass and function. This is particularly the case given that the updated EWGSOP2 recommendations mention that healthcare professionals have increasing possibilities for preventing, delaying, treating and even reversing sarcopenia.[38] EWGSOP2 have described that low muscle strength should be considered as "probable sarcopenia". Therefore, systematically measuring the handgrip of each COPD patient, independent of the clinical setting, can contribute to early detection, prevention and treatment of sarcopenia. Muscle strength is determined by the velocity and type of contraction, size and motor units activated, [7] which are affected early in COPD patients.[7] However, this does not mean that we should consider only handgrip for early detection of sarcopenia in patients with COPD, because the relationship between muscle mass and muscle function is nonlinear.[72] In addition, according to new recommendations of the Global Leadership Initiative on Malnutrition,[73] having low muscle mass with normal muscle strength would be considered to be a result of malnutrition rather than sarcopenia.

We propose that the EWGSOP2 algorithm be used in clinical practice,[38] and muscle strength assessed in all COPD patients. We call for more research directed towards defining optimal cut-off points of tests measuring muscle mass or function in patients with COPD. Importantly, multidisciplinary interventions such as pulmonary rehabilitation, which is included in the EWGSOP2 algorithm, have been shown to improve physical performance and exercise capacity in patients with COPD. The different steps of the diagnostic approach to sarcopenia in patients with COPD, encompassing case finding, assessment, confirmation and determining its severity, are illustrated in **Table 3**.

Finally, it is important to highlight that we found the overall prevalence of sarcopenia in COPD patients to be twice as high as compared to a healthy elderly population. In conclusion, sarcopenia defined as low muscle mass plus reduced muscle function is a frequent extrapulmonary condition in COPD patients. Despite the sarcopenia consensus criteria, there is still a lack of agreement in the optimal method and cut-off points to assess sarcopenia in general and in COPD patients in particular. Therefore, it is necessary to standardise the assessment methods for clinical practice and future research in COPD patients.

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https://err.ersjournals.com/content/28/154/190049

REFERENCES

1. Celli BR, Decramer M, Wedzicha JA, et al. An official American Thoracic Society/European Respiratory Society statement: research questions in COPD. Eur Respir Rev 2015; 24: 159–172.
2. Houben-Wilke S, Augustin IM, Vercoulen JH, et al. COPD stands for complex obstructive pulmonary disease. Eur Respir Rev 2018; 27: 180027.
3. 3Barnes PJ, Celli BR. Systemic manifestations and comorbidities of COPD. Eur Respir J 2009; 33: 1165–1185.
4. Jaitovich A, Barreiro E. Skeletal muscle dysfunction in chronic obstructive pulmonary disease what we know and can do for our patients. Am J Respir Crit Care Med 2018; 198: 175–186.
5. Agusti A, Soriano JB. COPD as a systemic disease. COPD 2008; 5: 133–138.
6. Rabe KF, Watz H. Chronic obstructive pulmonary disease. Lancet 2017; 389: 1931–1940.
7. Maltais F, Decramer M, Casaburi R, et al. An official American Thoracic Society/European Respiratory Society statement: update on limb muscle dysfunction in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2014; 189: e15–e62.
8. Buckinx F, Landi F, Cesari M, et al. Pitfalls in the measurement of muscle mass: a need for a reference standard. J Cachexia Sarcopenia Muscle 2018; 9: 269–278.
9. Hwang JA, Kim YS, Leem AY, et al. Clinical implications of sarcopenia on decreased bone density in men with COPD. Chest 2017; 151: 1018–1027.
10. van de Bool C, Gosker HR, van den Borst B, et al. Muscle quality is more impaired in sarcopenic patients with chronic obstructive pulmonary disease. J Am Med Dir Assoc 2016; 17: 415–420.
11. Lee IS, Leem AY, Lee SH, et al. Relationship between pulmonary function and bone mineral density in the Korean National Health and Nutrition Examination Survey. Korean J Intern Med 2016; 31: 899–909.
12. Costa TM, Costa FM, Moreira CA, et al. Sarcopenia in COPD: relationship with COPD severity and prognosis. J Bras Pneumol 2015; 41: 415–421.
13. Chung JH, Hwang HJ, Han CH, et al. Association between sarcopenia and metabolic syndrome in chronic obstructive pulmonary disease: the Korea National Health and Nutrition Examination Survey (KNHANES) from 2008 to 2011. COPD 2015; 12: 82–89.
14. Koo HK, Park JH, Park HK, et al. Conflicting role of sarcopenia and obesity in male patients with chronic obstructive pulmonary disease: Korean National Health and Nutrition Examination Survey. PLoS One 2014; 9: e110448.
15. Sergi G, Coin A, Marin S, et al. Body composition and resting energy expenditure in elderly male patients with chronic obstructive pulmonary disease. Respir Med 2006; 100: 1918–1924.
16. Joppa P, Tkacova R, Franssen FME, et al. Sarcopenic obesity, functional outcomes, and systemic inflammation in patients with chronic obstructive pulmonary disease. J Am Med Dir Assoc 2016; 17: 712–718
17. Pothirat C, Chaiwong W, Phetsuk N, et al. The relationship between body composition and clinical parameters in chronic obstructive pulmonary disease. J Med Assoc Thai 2016; 99: 386–393.
18. Gologanu D, Ionita D, Gartonea T, et al. Body composition in patients with chronic obstructive pulmonary disease. Maedica (Buchar) 2014; 9: 25–32.

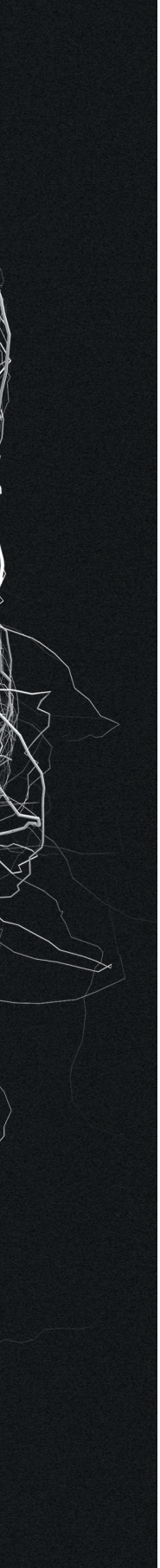
19. Rutten EPA, Calverley PMA, Casaburi R, et al. Changes in body composition in patients with chronic obstructive pulmonary disease: do they influence patient-related outcomes? Ann Nutr Metab 2013; 63: 239–247.
20. Verhage TL, Heijdra Y, Molema J, et al. Associations of muscle depletion with health status. Another gender difference in COPD? Clin Nutr 2011; 30: 332–338.
21. van Wetering CR, van Nooten FE, Mol SJM, et al. Systemic impairment in relation to disease burden in patients with moderate COPD eligible for a lifestyle program. Int J COPD 2008; 3: 443–451.
22. Vermeeren MAP, Creutzberg EC, Schols AMWJ, et al. Prevalence of nutritional depletion in a large out-patient population of patients with COPD. Respir Med 2006; 100: 1349–1355.
23. Steuten LMG, Creutzberg EC, Vrijhoef HJM, et al. COPD as a multicomponent disease: inventory of dyspnoea, underweight, obesity and fat free mass depletion in primary care. Prim Care Respir J 2006; 15: 84–91.
24. Vestbo J, Prescott E, Almdal T, et al. Body mass, fat-free body mass, and prognosis in patients with chronic obstructive pulmonary disease from a random population sample: findings from the Copenhagen City Heart Study. Am J Respir Crit Care Med 2006; 173: 79–83.
25. Barker BL, McKenna S, Mistry V, et al. Systemic and pulmonary inflammation is independent of skeletal muscle changes in patients with chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 2014; 9: 975–981.
26. Kim SB, Kang YA, Jung JY, et al. Body mass index and fat free mass index in obstructive lung disease in Korea. Int J Tuberc Lung Dis 2014; 18: 102–108.
27. Andersson M, Slinde F, Grönberg AM, et al. Physical activity level and its clinical correlates in chronic obstructive pulmonary disease: a cross-sectional study. Respir Res 2013; 14: 128.
28. Engelen MP, Schols AM, Lamers RJ, et al. Different patterns of chronic tissue wasting among patients with chronic obstructive pulmonary disease. Clin Nutr 1999; 18: 275–280.
29. Rutten EPA, Grydeland TB, Pillai SG, et al. Quantitative CT: associations between emphysema, airway wall thickness and body composition in COPD. Pulm Med 2011; 2011: 419328.
30. Celli BR, Locantore N, Tal-Singer R, et al. Emphysema and extrapulmonary tissue loss in COPD: a multi-organ loss of tissue phenotype. Eur Respir J 2018; 51: 1702146.
31. Spruit MA, Gosselink R, Troosters T, et al. Muscle force during an acute exacerbation in hospitalised patients with COPD and its relationship with CXCL8 and IGF-I. Thorax 2003; 58: 752–756.
32. Abdulai RM, Jensen TJ, Patel NR, et al. Deterioration of limb muscle function during acute exacerbation of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2018; 197: 433–449.
33. Muscaritoli M, Anker SD, Argiles J, et al. Consensus definition of sarcopenia, cachexia and pre-cachexia: joint document elaborated by Special Interest Groups (SIG) "cachexia-anorexia in chronic wasting diseases" and "nutrition in geriatrics". Clin Nutr 2010; 29: 154–159.
34. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al. Sarcopenia: European consensus on definition and diagnosis: report of the European Working Group on Sarcopenia in Older People. Age Ageing 2010; 39: 412–423.

35. Fielding RA, Vellas B, Evans WJ, et al. Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. J Am Med Dir Assoc 2011; 12: 249–256.
36. Studenski SA, Peters KW, Alley DE, et al. The FNIH sarcopenia project: rationale, study description, conference recommendations, and final estimates. J Gerontol A Biol Sci Med Sci 2014; 69: 547–558.
37. Chen LK, Liu LK, Woo J, et al. Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia. J Am Med Dir Assoc 2014; 15: 95–101.
38. Cruz-Jentoft AJ, Bahat G, Bauer J, et al. Sarcopenia: revised European consensus on definition and diagnosis. Age Ageing 2019; 48: 16–31.
39. Rosenberg IH. Sarcopenia: origins and clinical relevance. J Nutr 1997; 127: Suppl. 5, 990S–991S.
40. Cruz-Jentoft AJ, Sayer AA. Sarcopenia. Lancet 2019; 393: 2636–2646.
41. Lee LW, Lin CM, Li HC, et al. Body composition changes in male patients with chronic obstructive pulmonary disease: aging or disease process? PLoS One 2017; 12: e0180928 .
42. Bone AE, Hepgul N, Kon S, et al. Sarcopenia and frailty in chronic respiratory disease. Chron Respir Dis 2017; 14: 85–99.
43. Marty E, Liu Y, Samuel A, et al. A review of sarcopenia: enhancing awareness of an increasingly prevalent disease. Bone 2017; 105: 276–286.
44. Evans RA, Morgan MDL. The systemic nature of chronic lung disease. Clin Chest Med 2014; 35: 283–293.
45. Natanek SA, Gosker HR, Slot IGM, et al. Heterogeneity of quadriceps muscle phenotype in chronic obstructive pulmonary disease (COPD); implications for stratified medicine? Muscle Nerve 2013; 48: 488–497.
46. Nilwik R, Snijders T, Leenders M, et al. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. Exp Gerontol 2013; 48: 492–498.
47. Shafiee G, Keshtkar A, Soltani A, et al. Prevalence of sarcopenia in the world: a systematic review and meta-analysis of general population studies. J Diabetes Metab Disord 2017; 16: 21.
48. Cruz-Jentoft AJ, Landi F, Schneider SM, et al. Prevalence of and interventions for sarcopenia in ageing adults: a systematic review. Report of the International Sarcopenia Initiative (EWGSOP and IWGS). Age Ageing 2014; 43: 748–759.
49. Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. J Clin Epidemiol 2009; 62: e1–34.
50. Shamseer L, Moher D, Clarke M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. BMJ 2015; 350: g7647.
51. Hoy D, Brooks P, Woolf A, et al. Assessing risk of bias in prevalence studies: modification of an existing tool and evidence of interrater agreement. J Clin Epidemiol 2012; 65: 934–939.
52. Wells GA, Shea B, O'Connell D, et al. The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Non-randomised Studies in Meta-analyses. 2014. www.ohri.ca/programs/clinical_ epidemiology/oxford.asp. Date last accessed: April 05, 2019.

53. 53 Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002; 21: 1539–1558.
54. da Costa BR, Juni P. Systematic reviews and meta-analyses of randomized trials: principles and pitfalls. Eur Heart J 2014; 35: 3336–3345.
55. de Blasio F, Di Gregorio A, de Blasio F, et al. Malnutrition and sarcopenia assessment in patients with chronic obstructive pulmonary disease according to international diagnostic criteria, and evaluation of raw BIA variables. Respir Med 2018; 134: 1–5.
56. Munhoz da Rocha Lemos Costa T, Costa FM, Jonasson TH, et al. Body composition and sarcopenia in patients with chronic obstructive pulmonary disease. Endocrine 2018; 60: 95–102.
57. Limpawattana P, Inthasuwan P, Putraveephong S, et al. Sarcopenia in chronic obstructive pulmonary disease: a study of prevalence and associated factors in the Southeast Asian population. Chronic Respir Dis 2018; 15: 250–257.
58. Trajanoska K, Schoufour JD, Darweesh SKL, et al. Sarcopenia and its clinical correlates in the general population: the Rotterdam study. J Bone Miner Res 2018; 33: 1209–1218.
59. Byun MK, Cho EN, Chang J, et al. Sarcopenia correlates with systemic inflammation in COPD. Int J COPD 2017; 12: 669–675.
60. Lian J, Pan DZ, An XQ, et al. Changes in body composition and associated factors for sarcopenia in patients with chronic obstructive pulmonary disease. Chinese General Practice 2017; 20: 3504–3508.
61. Borda MG, Celis-Preciado CA, Pérez-Zepeda MU, et al. Sarcopenia en ancianos con antecedente de EPOC/asma: resultados del estudio SABE – Bogotá [Sarcopenia in the elderly with a history of COPD/asthma: results of the SABE-Bogotá study]. Rev Esp Geriatr Gerontol 2017; 52: 313–316.
62. Tasar PT, Sahin S, Karaman E, et al. Prevalence and risk factors of sarcopenia in elderly nursing home residents. Eur Geriatr Med 2015; 6: 214–219.
63. Jones SE, Maddocks M, Kon SSC, et al. Sarcopenia in COPD: prevalence, clinical correlates and response to pulmonary rehabilitation. Thorax 2015; 70: 213–218.
64. Landi F, Liperoti R, Fusco D, et al. Sarcopenia and mortality among older nursing home residents. J Am Med Dir Assoc 2012; 13: 121–126.
65. Al Haddad MA, John M, Hussain S, et al. Role of the timed up and go test in patients with chronic obstructive pulmonary disease. J Cardiopulm Rehabil Prev 2016; 36: 49–55.
66. Mesquita R, Wilke S, Smid DE, et al. Measurement properties of the timed up & go test in patients with COPD. Chron Respir Dis 2016; 13: 344–352.
67. DePew ZS, Karpman C, Novotny PJ, et al. Correlations between gait speed, 6-minute walk distance, physical activity, and self-efficacy in patients with severe chronic lung disease. Respir Care 2013; 58: 2113–2119.
68. Ciciliot S, Rossi AC, Dyar KA, et al. Muscle type and fiber type specificity in muscle wasting. Int J Biochem Cell Biol 2013; 45: 2191–2199.
69. Correa-de-Araujo R, Harris-Love MO, Miljkovic I, et al. The need for standardized assessment of muscle quality in skeletal muscle function deficit and other aging-related muscle dysfunctions: a symposium report. Front Physiol 2017; 8: 87.
70. Rutten EPA, Spruit MA, Wouters EFM. Critical view on diagnosing muscle wasting by single-frequency bio-electrical impedance in COPD. Respir Med 2010; 104: 91–98.

71. Volgyi E, Tylavsky FA, Lyytikainen A, et al. Assessing body composition with DXA and bioimpedance: effects of obesity, physical activity, and age. Obesity (Silver Spring) 2008; 16: 700–705.
72. Shrikrishna D, Patel M, Tanner RJ, et al. Quadriceps wasting and physical inactivity in patients with COPD. Eur Respir J 2012; 40: 1115–1122.
73. Cederholm T, Jensen GL, Correia M, et al. GLIM criteria for the diagnosis of malnutrition – a consensus report from the global nutrition community. Clin Nutr 2019; 38: 1-9.





3.4

Nonalcoholic Fatty Liver Disease in The Rotterdam Study: About Muscle Mass, Sarcopenia, Fat Mass, and Fat Distribution

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide. Obesity is a major risk factor for NAFLD and recently, low skeletal muscle mass emerged as additional risk factor for NAFLD. However, the different contributions of body mass index (BMI) to the risk of NAFLD are not yet well-known. We therefore studied body composition and muscle function with NAFLD in an elderly population-based study. Participants of European descent underwent dual-energy X-ray absorptiometry (DXA) and hepatic ultrasonography. NAFLD was defined as liver steatosis in absence of secondary causes for steatosis. Skeletal muscle index (SMI) was defined as appendicular lean mass/height^2 and (pre)sarcopenia was defined using the European Working Group on Sarcopenia in Older People (EWGSOP) consensus guidelines. All analyses were stratified by sex and BMI (cut point: 25 kg/m^2) and adjusted for age, weight, height, homeostasis model assessment of insulin resistance (HOMA-IR), triglycerides, and android-fat-to-gynoid-fat ratio (AGR). We included 4,609 participants, of whom 1,623 had NAFLD (n = 161 normal-weight and n = 1462 overweight). Presarcopenia and sarcopenia prevalence was low (5.9% and 4.5%, respectively) and both were not associated with NAFLD. SMI was associated with less NAFLD in normal-weight women (OR, 0.48; 95% CI, 0.29 to 0.80). A similar association for SMI and NAFLD was seen in normal-weight men, but significance dissipated after adjustment for AGR (OR, 0.63; 95% CI, 0.39 to 1.02). Generally, fat mass was a better predictor for NAFLD than lean mass. In particular, android fat mass was associated with all NAFLD subgroups (OR from 1.77 in overweight men to 8.34 in normal-weight women, P_{max} = 0.001), whereas substitution of gynoid fat mass for other body components had a significant protective association with NAFLD in every subgroup, but normal-weight men. Likewise, AGR was the best performing predictor for NAFLD prevalence (OR from 1.97 in normal-weight men to 4.81 in normal-weight women, P_{max} < 0.001). In conclusion, both high fat mass and low SMI were associated with normal-weight NAFLD. However, fat distribution (as assessed by AGR) could best predict NAFLD prevalence.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease today and it parallels the epidemic of obesity and diabetes mellitus.[1,2] NAFLD constitutes a major public health threat because it leads not only to an increased risk of liver-specific,[3] but also cardiovascular, morbidity and mortality.

Obesity is strongly related to NAFLD.[1] That said, certainly not all obese individuals have NAFLD and not every NAFLD patient is obese. In fact, about one in six of the NAFLD patients have a normal body mass index (BMI).[4] Therefore, the accuracy of BMI as the all-encompassing measure of adiposity is debated.[5] Presence of excess (visceral) fat mass is a well-established risk factor for NAFLD, independent of BMI.[6, 7] Recently, emerging evidence suggested that low muscle mass (or presarcopenia) also contributes to the risk of NAFLD.[8–10] Indeed, because skeletal muscle mass is the primary tissue responsible for insulin-mediated glucose disposal, skeletal muscle plays an important role in glucose homeostasis and insulin resistance, which are key in the pathogenesis of NAFLD.[11]

However, evidence on the association between skeletal muscle mass and NAFLD predominantly originates from young Asian populations with a high BMI.[9,12] This gives room for thought whether it is a true shortage of muscle mass, or a relative excess of fat mass, or both that is associated with NAFLD. Interestingly, a recent population-based study found that the two body components, lean body mass and fat mass, both explained the relation between BMI and mortality.[13] To the best of our knowledge, there are no studies that have examined the role of both components, independent of each other, in relation to NAFLD. But considering the above, there are arguments to think that both contributions of BMI, ie, (lean) muscle mass and fat mass, are risk factors for NAFLD.

We therefore studied the independent association of the different components of the body with NAFLD, stratified by sex and BMI, in a large elderly European population. We were particularly interested in the association between skeletal muscle mass with (sarcopenia) or without (presarcopenia) loss of muscle function, and NAFLD. In addition, we assessed which body composition parameter could best predict NAFLD prevalence.

SUBJECTS AND METHODS

Study Population

This cross-sectional analysis included participants from The Rotterdam Study, a large ongoing population-based cohort of participants aged 45 years or older in the Neth-

erlands. The design and rationale of this population-based study have been described in detail previously. [14] In short, the study commenced in 1989 and comprises three cohorts. Hepatic imaging has been part of the physical workup since 2009. There are no specific eligibility criteria for The Rotterdam Study, except for the minimum age and residential area (ZIP codes). As body composition differs among ethnicities [12] and The Rotterdam Study is predominantly of European background, the low number of non-European participants were excluded to examine a homogenous population. Ethnicity was determined using genome-wide genotypes into HapMap CEU release 22 (build 36). The genotype data was pruned to end up with variants in linkage equilibrium and the ancestry component for each individual was estimated on the basis of the maximum likelihood using the ADMIXTURE software.[14] The Rotterdam Study is approved by the institutional review board of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare and Sports. Written informed consent was obtained from all participants.

Dual-energy X-ray Absorptiometry Scanning

Body Components

Body components were assessed using dual-energy X-ray absorptiometry (DXA) scans with iDXA total body fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). All scans were analyzed using enCORE software, which divides scan results into total lean mass, total fat mass, and bone/organ mass. Total lean mass is the sum of trunk lean mass and appendicular lean mass (ALM; the sum of lean tissue from the arms and legs), and total body fat mass is the sum of android fat mass (localized around the waist), gynoid fat mass (localized around the breasts, hips, and thighs), and fat mass not otherwise specified. The latter includes mainly trunk fat mass and a small proportion of appendicular fat mass, but as this was not further specified, we refer to this remaining component as trunk fat mass. The relative proportion (or fraction) of each component to the body was expressed as percentage of total body weight. For instance, ALM-fraction was calculated as ALM/total body weight*100.

Skeletal Muscle Mass

Skeletal muscle mass was estimated by the skeletal muscle index (SMI) using ALM divided by squared body height (kg/m^2), to adjust for variation in skeletal size. Low SMI was defined as ≤7.25 kg/m^2 in men and ≤5.67 kg/m^2 in women, based on cutoff values suggested by the European Working Group on Sarcopenia in Older People (EWGSOP).[15]

Fat Distribution

Fat distribution was assessed using the standardized android- fat-to-gynoid-fat ratio (AGR).

Presarcopenia and Sarcopenia

Grip Strength

Grip strength (proxy for overall muscle strength) was examined using a hydraulic hand dynamometer (Fabrication Enterprises Inc., USA). The maximum grip strength was defined as the maximum value (in kg) out of three serial attempts using the non-dominant hand. EWGSOP cutoff values for low grip strength were ≤29 kg for BMI ≤24 kg/m^2, ≤30 kg for BMI 24.1 to 28 kg/m^2, or ≤32 kg for BMI >28 kg/m^2 in men; and ≤17 kg for BMI ≤23 kg/m^2, ≤17.3 kg for BMI 23.1 to 26 kg/m^2, ≤18 kg for BMI 26.1 to 29 kg/m^2, or ≤21 kg for BMI >29 kg/m^2 in women.[15]

Gait Speed

Gait speed (measure of physical performance)[16] was examined using the GAITRite walkway (CIR Systems, Inc., Sparta, NJ, USA), 5.79-meter-long electronic walkway. Again, EWGSOP cutoffs were applied for the definition of low gait speed, ie, for men: <0.65 m/s if height ≤173 cm, or <0.76 m/s if height >173 cm; and for women: <0.65 m/s if height ≤159 cm, or <0.76 m/s if height >159 cm.[15]

Presarcopenia and Sarcopenia

Presarcopenia and sarcopenia were defined according to the EWGSOP consensus guideline.[15,17] Presarcopenia was defined as presence of low SMI, and sarcopenia as low SMI plus either low muscle strength or low physical performance.

Hepatic Imaging

Hepatic Steatosis

Hepatic steatosis was assessed using abdominal ultrasound (US), which was carried out by a certified and experienced technician (Ultrasonography, Hitachi HI VISION 900). Images were stored digitally. Diagnosis of steatosis was determined dichotomously as presence of a hyperechogenic liver parenchyma.[18] Participants with secondary causes for steatosis were excluded from this study, ie, those with (i) excessive alcohol consumption (men >30 g/day and women >20 g/day); (ii) use of steatogenic drugs, ie, amiodarone, systemic corticosteroids, methotrexate, or tamoxifen (extracted from automated pharmacy linkage); and (iii) viral hepatitis, based on hepatitis B surface antigen and anti-hepatitis C virus, measured by an automatic immunoassay (Roche

Diagnostic GmbH, Mannheim, DE). The remainder participants with steatosis were considered to have NAFLD.

NAFLD Severity

NAFLD severity was assessed using transient elastography (FibroScan®, EchoSens, Paris, France). Practical implementation of this examination has been described in detail previously.[19] Liver stiffness measurements (LSM, in kilopascals [kPa]) were available for a subset of the study population (from January 2011 onward). A single operator obtained 10 serial measurements using either the M or XL-probe dependent on the thickness of the subcutaneous fat layer. We excluded participants with (i) unreliable measurements (ie, interquartile range/median LSM >0.3 kPa and LSM ≥ 7.1 kPa)[20]; (ii) failure of assessment; or (iii) presence of an intracardial device. For this study, non-alcoholic steatohepatitis (NASH), or advanced NAFLD, was defined as presence of steatosis and LSM ≥ 8.0 kPa, a proxy for fibrosis.[21]

Biochemistry and Additional Covariates

Fasting blood lipids, platelet count, glucose, alanine aminotransferase (ALT), aspartate aminotransferase, and gamma-glutamyl-transferase were measured using automatic enzyme procedures (Roche Diagnostic GmbH, Mannheim, DE). Insulin was determined using an automatic immunoassay (Roche Diagnostic GmbH).

Data on demographics, physical activity, smoking behavior, educational level, and comorbid conditions were obtained during an extensive home interview. Daily energy intake in kilocalories (kcal) and alcohol intake in grams was assessed using a 389-item semiquantitative food frequency questionnaire.[22] We excluded unreliable energy consumption of <500 or >7500 kcal/day. Blood pressure measurements were obtained using two subsequent measurements in upright position. BMI was calculated as weight/ height^2 (kg/m^2) and dichotomized into normal-weight: <25 kg/m^2 and overweight: ≥25 kg/m^2.

Insulin resistance was determined using the homeostasis model assessment of insulin resistance (HOMA-IR = fasting glucose (mmol/dL) times fasting insulin (mU/L) divided by 22.5).[23] The metabolic syndrome was diagnosed when three out of five metabolic traits were present: (i) abdominal obesity, ie, waist circumference >102 cm in men and >88 cm in women; (ii) serum triglycerides ≥130 mg/dL or drug treatment for elevated triglycerides; (iii) serum high-density lipoprotein cholesterol (HDL-C) <40 mg/dL in men and <50 mg/dL in women or drug treatment for low HDL-C; (iv) blood pressure ≥130/85 mm Hg or drug treatment for elevated blood pressure; (v) fasting plasma glucose ≥100 mg/dL or drug treatment for elevated blood glucose. [(24)] Hypertension was diagnosed if either systolic (≥140 mm Hg) or diastolic (≥90 mm Hg) blood pressure was increased and/or if the participant was on antihypertensive

medication. Diabetes was diagnosed as fasting glucose above 7.0 mmol/L and/or drug treatment for elevated blood glucose.

STATISTICAL ANALYSES

To reduce bias due to missing data, missing values were imputed using multiple imputation (fully conditioned specification). Details on this imputation process can be found in the Supporting Methods. Continuous data were presented as mean ± standard deviation (SD) or median with 25th or 75th percentile (P25 to P75). Categorical data were presented as percentage. The Chi-square test, one-way analyses of variance, or the Kruskal-Wallis test were used to assess differences by strata.

Associations between body composition and NAFLD were examined using logistic regression models stratified by BMI and sex, because of the known sex differences in body composition. [25] We examined the association between (i) the different body components, (ii) SMI, and (iii) (pre)sarcopenia with NAFLD. We evaluated weight, height, weight and height, BMI, and body fat fraction as adjustment for body composition using the Akaike Information Criterion (AIC).[26] After evaluation, models adjusted for weight and height performed best. Moreover, without weight as factor in the model, all body components were associated with higher prevalence of NAFLD. If body fat fraction was put in the model together with ALM (or SMI), the beta for ALM could reflect two scenarios. First, the beta could reflect an increase in ALM and subsequent increase in total body weight. In this case, total body fat would increase too (as body fat fraction is set and total weight increases). Second, the beta could reflect an increase of ALM at a set weight, while body fat fraction remains the same (weight is set) and bone and organ mass hardly varies. Thus, an increase of ALM would then be at the expense of trunk lean mass. The latter scenario reflects the substitution of one component of the body at the expense of another. We performed such a substitution analyses,[27,28] which is often used in nutritional epidemiology to assess the relative replacement of one nutrient by another for a given caloric intake. A similar formula can be applied to assess the relative replacement of one body component with another for a given body weight. For example:

NAFLD ~ total lean mass (%) + bone / organ mass (%) + total body weight (kg)

In this example, only total fat mass (%) is not included in this formula and hence, the beta for total lean mass reflects the increase of total lean mass at the expense of total body fat mass (in % body weight).

In addition to weight and height, all analyses were adjusted for age and study cohorts as well. Potential confounding of a nested set of sociodemographic, lifestyle, and metabolic predictor variables (based on the literature[2]) was tested, taking potential overfitting into account.

Furthermore, to assess which parameter had the best performance for NAFLD (i.e., explained more variation of the outcome and thus resulted in a better model fit), we compared SMI, ALM-fraction, AGR, body fat fraction, (pre)sarcopenia, grip, and speed using the AIC.

To test the robustness of our conclusions we performed three sensitivity analyses. First, we explored ALM-fraction as alternative proxy for skeletal muscle mass to facilitate comparison with previous reports. [9,10] Second, as gait speed measurements were performed on a separate day at the research facility, this measurement was missing in 32.6% of individuals. In the main analysis, we assumed that if one of both proxies for physical functioning was normal, there would be no sarcopenia. In the sensitivity analysis, we used imputed grip strength and gait speed data to reclassify sarcopenia. Third, we analyzed the association of SMI with NASH to assess whether SMI was also associated with NAFLD severity.

We checked all analyses for potential multicollinearity using the variance inflation factor. In addition, we corrected for the inflated type I error that arises due to multiple testing. We applied the adapted method proposed by Sidák,[29,30] using the effective number of tests instead of the actual number of tests (n = 8). This adaptation took into account that the different body components are strongly interrelated and, hence, are not independent from each other. The corrected significance level was $P < 0.010$. All analyses were performed using R version 3.5.1 (R core team, Vienna, Austria).

RESULTS

Study Population

The flowchart of the study is illustrated in Fig. 1. In total, 5967 participants underwent abdominal US. We excluded 253 participants (4.2%) because of unreliable food questionnaires, missing data on DXA scans or outlier values. Second, 887 participants (15.5%) were excluded while having secondary causes for steatosis. Last, 218 participants were excluded because of non-European background. Hence, the total number of eligible study participants was 4609. Mean age was 69.3 ± 9.2 years, 57.0% was female and mean BMI was 27.5 ± 4.2 kg/m^2 (range, 15.0 to 47.2 kg/m^2). Both original and imputed data of the total population are depicted in **Supporting Table 1**.

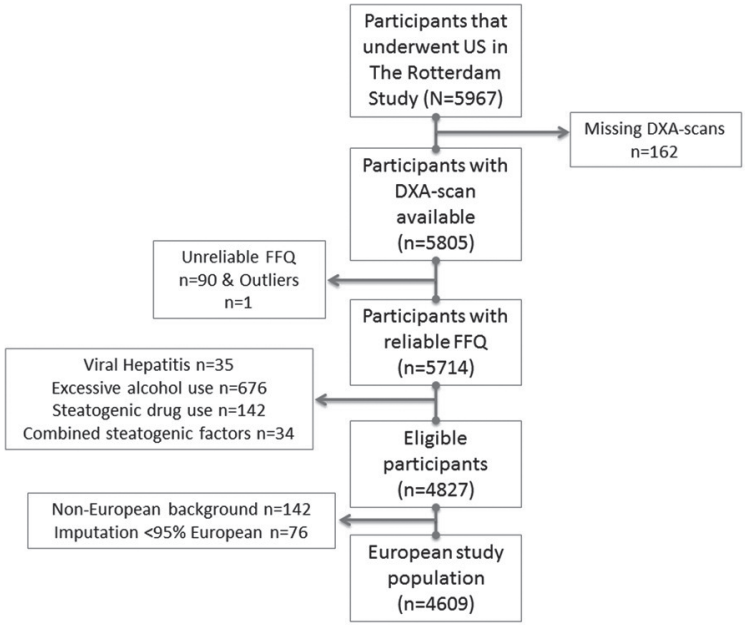


Figure1 | Flowchart of the study. DXA = dual-energy X-ray absorptiometry; FFQ = food frequency questionnaire; US = ultrasound.

NAFLD Characteristics

The overall prevalence of NAFLD in this study population was 35.2% (n = 1623). Of those, 161 individuals were normal-weight (10%) and 1462 were overweight (90%). Data on demographics, biochemistry, and comorbidities are given, stratified by BMI, in **Supporting Table 2**. Differences between NAFLD strata were most pronounced in the overweight group. However, ALT, gamma-glutamyltransferase, insulin resistance, and triglycerides were significantly different in both NAFLD subgroups.

Body Composition and NAFLD

Table 1 depicts the data on body composition stratified by sex and BMI. Fat mass parameters were higher in every NAFLD subgroup, whereas lean mass parameters were lower mostly in the normal-weight NAFLD subgroups. Interestingly, ALM- fraction was the only parameter that was lower in every NAFLD subgroup. Also, overall prevalence of presarcopenia and sarcopenia was low (5.9% and 4.5%, respectively) and not associated with NAFLD (**Table 1**).

We observed no clear effect modification, but the association between body components and NAFLD was most pronounced in normal-weight women (**Table 2**). Again, fat mass was generally associated with higher NAFLD and lean mass with lower NAFLD prevalence. In both sexes, android fat mass remained associated with NAFLD.

Table 1 | Study Characteristics of Participants for Different NAFLD Strata

	Normal weight (n= 1,339)			**Overweight (n= 3,270)**		
	Men (n= 1,980)					
	No NAFLD (n= 432)	NAFLD (n= 67)	P*	No NAFLD (n= 822)	NAFLD (n= 659)	P*
Height (m)	177.0 (7.1)	176.8 (7.8)	0.796	176 (7.1)	176 (6.9)	0.703
Weight (kg)	72.9 (7.0)	74.8 (7.6)	0.041	86.8 (10.1)	93.2 (12.9)	<0.001
Total fat mass (kg)	19.1 [16.5. 21.8]	21.0 [18.6, 24.1]	<0.001	26.6 [23.3, 30.5]	30.9 [26.6, 36.3]	<0.001
Body fat fraction (%)	26.0 (4.6)	28.6 (4.3)	<0.001	31.4 (4.6)	34.3 (4.6)	<0.001
Android fat mass (kg)	1.70 [1.28, 2.08]	2.01 [1.75, 2.42]	<0.001	2.73 [2.21, 3.24]	3.40 [2.82, 4.08]	<0.001
Trunk/appendicular fat mass (kg)	14.9 [12.8, 16.8]	16.2 [14.6, 18.5]	<0.001	20.4 [18.0, 23.3]	23.6 [20.5, 27.7]	<0.001
Gynoid fat mass (kg)	2.56 [2.19, 2.95]	2.71 [2.35, 3.08]	0.028	3.38 [2.94, 3.98]	3.86 [3.22, 4.65]	<0.001
AGR	0.66 [0.54, 0.76]	0.76 [0.68, 0.84]	0.028	0.79 [0.69, 0.89]	0.87 [0.79, 0.97]	<0.001
Total lean mass (kg)	51.0 [47.4, 54.8]	49.9 [47.0, 54.5]	0.371	55.7 [51.8, 59.9]	57.0 [52.9, 61.2]	<0.001
Trunk lean mass (kg)	27.6 [26.0, 29.6]	26.9 [25.5, 28.9]	0.209	29.5 [27.6, 31.6]	30.1 [28.0, 32.4]	0.001
Appendicular lean mass (kg)	23.3 [21.1, 25.4]	23.1 [20.8, 25.2]	0.998	26.0 [24.0, 28.7]	26.9 [24.7, 29.4]	<0.001
SMI (kg/m^2)	7.43 (0.74)	7.41 (0.69)	0.769	8.45 (0.80)	8.70 (0.91)	<0.001
ALM-fraction (%)	31.9 [30.3, 33.9]	31.2 [29.1, 33.2]	0.015	30.5 [28.7, 32.3]	29.4 [27.7, 31.0]	<0.001
Normal SMI	58.8	58.2	0.63	94.3	94.7	0.337
Presarcopenia	24.5	20.9		3	2	
Sarcopenia	16.7	20.9		2.7	3.3	
Gait speed (m/s)	1.26 [1.11,1.40]	1.27 [1.13, 1.38]	0.727	1.24 [1.09, 1.35]	1.23 [1.09. 1.34]	0.99
Hand grip strength (kg)	35.4 (8.5)	33.0 (9.2)	0.036	36.8 (8.9)	36.7 (9.1)	0.841

Table 1 | Study Characteristics of Participants for Different NAFLD Strata (*continued*)

	Normal weight (n= 1,339)			Overweight (n= 3,270)		
	Women (n= 2,629)					
	No NAFLD (n=432)	NAFLD (n=67)	P*	No NAFLD (n=822)	NAFLD (n=659)	P*
Height (m)	164.0 (6.6)	163.4 (6.4)	0.42	162.5 (6.7)	162.7 (6.4)	0.435
Weight (kg)	61.0 (6.6)	63.1 (5.6)	0.003	75.8 (10.5)	82.6 (11.7)	<0.001
Total fat mass (kg)	20.9 [17.7, 23.4]	23.5 [21.1, 25.0]	<0.001	29.9 [26.5, 34.5]	34.8 [30.3, 40.8]	<0.001
Body fat fraction (%)	34.1 (4.9)	37.4 (3.6)	<0.001	41.8 (4.4)	44.3 (4.3)	<0.001
Android fat mass (kg)	1.48 [1.12, 1.81]	1.95 [1.70, 2.29]	<0.001	2.47 [2.06, 2.99]	3.20 [2.67, 3.92]	<0.001
Trunk/Appendicular fat mass (kg)	16.1 [13.7, 17.9]	17.9 [16.4, 19.0]	<0.001	22.7 [20.2, 26.4]	26.5 [23.1, 30.9]	<0.001
Gynoid fat mass (kg)	3.33 [2.81, 3.83]	3.46 [2.97, 3.86]	0.08	4.67 [3.99, 5.48]	5.16 [4.35, 6.13]	<0.001
AGR	0.43 [0.35, 0.51]	0.56 [0.50, 0.65]	0.08	0.53 [0.46, 0.61]	0.62 [0.55, 0.71]	<0.001
Total lean mass (kg)	37.3 [34.7, 40.1]	37.1 [34.4, 38.7]	0.118	40.3 [37.6, 43.7]	42.3 [39.3, 45.8]	<0.001
Trunk lean mass (kg)	20.8 [19.4, 22.3]	20.5 [19.2, 21.8]	0.135	21.9 [20.6, 23.7]	22.9 [21.3, 24.8]	<0.001
Appendicular lean mass (kg)	16.5 [15.0, 18.1]	16.2 [15.2, 17.3]	0.109	18.3 [16.8, 20.2]	19.3 [17.6, 21.3]	<0.001
SMI (kg/m2)	6.14 (0.60)	6.03 (0.45)	0.088	7.01 (0.71)	7.36 (0.84)	<0.001
ALM-fraction (%)	27.7 [25.8, 29.4]	26.2 [24.7, 27.5]	<0.001	25.2 [23.7, 26.6]	24.2 [22.9, 25.5]	<0.001
Normal SMI	79.9	76.6	0.727	98.8	98.8	0.691
Presarcopenia	12.2	14.9		0.5	0.7	
Sarcopenia	7.9	8.5		0.7	0.5	
Gait speed (m/s)	1.24 [1.11, 1.36]	1.21 [1.08, 1.31]	0.994	1.17 [1.03, 1.28]	1.15 [1.02, 1.27]	0.57
Hand grip strength (kg)	21.8 (6.0)	20.9 (5.1)	0.176	21.77 (5.75)	21.61 (5.66)	0.553

Pooled data based on 74 imputations is presented as mean (SD), median [P25, P75], or percentage. Data is presented stratified by sex and BMI. NAFLD =nonalcoholic fatty liver disease; AGR =android-fat-to-gynoid-fat ratio; SMI =skeletal muscle index; ALM =appendicular lean mass; BMI =body mass index. Values of p are calculated using analyses of variance, Kruskal-Wallis, or the chi-square test.

Table 2 | Association of Different Parts of Body With NAFLD

	Men			
	Normal-weight (n= 499)	**P**	**Overweight (n= 1,481)**	**P**
Model 1				
Total lean mass	0.87 (0.80–0.96)	0.003	0.93 (0.90–0.96)	<0.001
Appendicular lean mass	0.80 (0.69–0.93)	0.003	0.91 (0.86–0.96)	0.001
Trunk lean mass	0.86 (0.74–1.00)	0.045	0.90 (0.85–0.95)	<0.001
Total body fat	1.16 (1.06–1.27)	0.001	1.08 (1.05–1.12)	<0.001
Trunk fat mass	1.22 (1.08–1.37)	0.001	1.11 (1.06–1.17)	<0.001
Gynoid fat mass	1.46 (0.81–2.63)	0.203	0.97 (0.80–1.17)	0.72
Android fat mass	3.49 (1.95–6.22)	<0.001	2.49 (2.00–3.11)	<0.001
Model 2				
Total lean mass	0.90 (0.82–0.99)	0.026	0.96 (0.93–1.00)	0.038
Appendicular lean mass	0.84 (0.72–0.97)	0.02	0.95 (0.89–1.01)	0.105
Trunk lean mass	0.90 (0.77–1.06)	0.199	0.94 (0.89–1.00)	0.065
Total body fat	1.13 (1.03–1.24)	0.011	1.04 (1.01–1.08)	0.022
Trunk fat mass	1.17 (1.04–1.33)	0.012	1.06 (1.01–1.11)	0.03
Gynoid fat mass	1.32 (0.71–2.42)	0.377	0.96 (0.78–1.18)	0.685
Android fat mass	2.94 (1.59–5.45)	0.001	1.77 (1.40–2.25)	<0.001
Model 3				
Total lean mass	0.94 (0.85–1.04)	0.202	0.97 (0.94–1.01)	0.147
Appendicular lean mass	0.87 (0.74–1.01)	0.071	0.97 (0.91–1.03)	0.296
Trunk lean mass	0.99 (0.83–1.17)	0.867	0.96 (0.90–1.02)	0.169
Total body fat	1.09 (0.99–1.20)	0.094	1.03 (1.00–1.07)	0.094
	Women			
	Normal-weight (n= 840)	**P**	**Overweight (n= 1,789)**	**P**
Model 1				
Total lean mass	0.79 (0.71–0.87)	<0.001	0.97 (0.93–1.01)	0.098
Appendicular lean mass	0.68 (0.57–0.81)	<0.001	0.95 (0.88–1.02)	0.146
Trunk lean mass	0.76 (0.64–0.87)	0.001	0.97 (0.91–1.02)	0.227
Total body fat	1.22 (1.10–1.35)	<0.001	1.04 (1.00–1.08)	0.034
Trunk fat mass	1.30 (1.14–1.49)	<0.001	1.06 (1.01–1.12)	0.02
Gynoid fat mass	0.73 (0.47–1.12)	0.15	0.63 (0.54–0.73)	<0.001
Android fat mass	12.5 (6.28–24.9)	<0.001	3.40 (2.71–4.27)	<0.001
Model 2				
Total lean mass	0.80 (0.71–0.89)	<0.001	0.96 (0.92–1.00)	0.074
Appendicular lean mass	0.69 (0.57–0.83)	<0.001	0.98 (0.90–1.06)	0.545
Trunk lean mass	0.76 (0.64–0.90)	0.001	0.93 (0.88–1.00)	0.035
Total body fat	1.20 (1.08–1.33)	0.001	1.05 (1.01–1.10)	0.02
Trunk fat mass	1.26 (1.09–1.44)	0.001	1.07 (1.01–1.13)	0.021

Table 2 | Association of Different Parts of Body With NAFLD (*continued*)

	Women			
	Normal-weight (n= 840)	**P**	**Overweight (n= 1,789)**	**P**
Gynoid fat mass	1.00 (0.62–1.61)	0.996	0.85 (0.73–1.00)	0.05
Android fat mass	8.34 (3.95–17.6)	<0.001	2.16 (1.69–2.76)	<0.001
Model 3				
Total lean mass	0.84 (0.75–0.94)	0.002	0.97 (0.93–1.01)	0.17
Appendicular lean mass	0.75 (0.62–0.91)	0.003	1.01 (0.94–1.10)	0.772
Trunk lean mass	0.82 (0.69–0.98)	0.029	0.93 (0.87–0.99)	0.025
Total body fat	1.16 (1.03–1.29)	0.011	1.06 (1.01–1.10)	0.01

Results are given as OR (95% CI) for NAFLD as outcome stratified by sex and BMI. Results in bold reflect significant findings with a P value <0.010. Model 1: adjusted for age, study cohorts, weight, and height. Model 2: in addition: HOMA-IR, and triglycerides. Model 3: in addition, AGR. Additional adjustments for confounding by education level, physical activity, alcohol intake, energy intake, ALT, and smoking resulted in negligible changes in odds ratio (<5%).
NAFLD = non-alcoholic fatty liver disease; OR: odds ratio; CI = confidence interval; BMI: body mass index; AGR = android-fat-to-gynoid-fat ratio; HOMA-IR = homeostasis model assessment of insulin resistance.

In addition, ALM remained independently associated with normal-weight women (OR, 0.75; 95% CI, 0.62 to 0.91; = 0.003; **Table 2**).

Supporting Table 3 shows the substitution analyses. Substituting ALM for fat mass was associated with lower prevalence of NAFLD, except when replaced for gynoid fat. Indeed, replacing components by gynoid fat mass was associated with lower NAFLD prevalence, whereas the opposite was true for android fat mass.

Comparing the performance of the different body composition parameters for NAFLD, fat mass parameters explained more variation of the outcome (resulted in a better model fit) than lean mass parameters (as indicated by a lower AIC). AGR was the predictor with the overall best model fit (**Supporting Table 4**). Interestingly, SMI and ALM-fraction performed equally with regard to the model fit in the analysis for NAFLD.

SMI and NAFLD

Table 3 shows the association between our exposure variable of interest, SMI, and NAFLD. SMI was associated with less NAFLD prevalence in normal-weight men, but was not significant after correction for multiple testing and adjustment for AGR. In normal-weight women, however, SMI remained associated with less NAFLD after full adjustment (OR, 0.48; 95%CI, 0.29 to 0.80; P = 0.005; Table 3). Using ALM-fraction (instead of SMI) provided similar conclusions, underscoring the robustness of our results (Supporting Table 5). No independent association was found between SMI and overweight NAFLD. However, SMI was relatively high in this population. The prevalence of low SMI (ie, ≤7.25 kg/m^{2}or ≤5.67 kg/m^2) was only 3% in overweight

Table 3 | SMI and NAFLD

		Men			
		Normal-weight (n= 499)	**P**	**Overweight (n = 1,481)**	**P**
Model 1	SMI	0.50 (0.32–0.79)	0.003	0.75 (0.63–0.90)	0.002
Model2	SMI	0.57 (0.36–0.91)	0.018	0.88 (0.72–1.06)	0.169
Model3	SMI	0.63 (0.39–1.02)	0.061	0.92 (0.76–1.12)	0.401
		Women			
		Normal-weight (n= 840)	**P**	**Overweight (n= 1789)**	**P**
Model 1	SMI	0.37 (0.23–0.59)	<0.001	0.90 (0.75–1.09)	0.291
Model 2	SMI	0.39 (0.23–0.64)	<0.001	0.98 (0.80–1.20)	0.846
Model 3	SMI	0.48 (0.29–0.80)	0.005	1.08 (0.87–1.33)	0.485

Results are given as OR (95% CI) for NAFLD as outcome stratified by sex and BMI. Results in bold reflect significant findings with a P value <0.010. Model 1: adjusted for age, study cohorts, weight and height. Model 2: in addition: HOMA-IR, and triglycerides. Model 3: in addition: AGR. Additional adjustments for confounding by education level, physical activity, alcohol intake, energy intake, ALT, and smoking resulted in negligible changes in odds ratio (<5%).
SMI = skeletal muscle index; NAFLD = non-alcoholic fatty liver disease; OR = odds ratio; CI = confidence interval; BMI = body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; AGR = android-fat-to-gynoid-fat ratio.

individuals against 28% in the normal-weight. Prevalence of (pre)sarcopenic obesity (ie, BMI≥30 kg/m^2) was even lower, in fact, only five out of the 1091obese individuals had a low SMI. Interestingly, four of them had NAFLD.

The analysis of presarcopenia and sarcopenia is given in Supporting Table 6. In model 1, sarcopenia was associated with NAFLD in men and presarcopenia with NAFLD in women, nonetheless these findings did not hold after further adjustment (Supporting Table 6). In total, 1548 participants (33.6%) had either missing data on grip strength or gait speed. None of the participants had missing data on both variables. Sensitivity analysis using imputed values on grip strength and gait speed resulted in reclassification of 24 participants and yielded similar results to the original data (data not shown).

Of the 1623 individuals with NAFLD, 1126 (69.4%) had data available on LSM. NASH prevalence was low (total population: n = 115, 10.2%; normal-weight: n = 10, 8.5%; and overweight: n = 105, 10.4%). The small number of cases in the normal-weight (three in men and seven in women) hampered the possibility to stratify by BMI. Supporting Table 7 shows that SMI was associated with lower NASH prevalence in women, but this was no longer significant after correction for multiple testing (OR, 0.48; 95% CI, 0.25 to 0.92; P = 0.027).

DISCUSSION

In this large Western population-based study we examined the association between muscle mass, fat mass, fat distribution, and NAFLD, using DXA scans and hepatic imaging, both highly reliable measuring methods. Moreover, this is the first study that examines the sarcopenia definition, as proposed by the EWGSOP consensus, in association with NAFLD. We made several novel observations. First, we showed that incremental skeletal muscle mass was consistently associated with lower NAFLD prevalence and severity in normal-weight women (using different approaches). This association was independent of metabolic confounders, and importantly, independent of fat distribution. A similar association was seen in normal-weight men, but significance did not hold after correction for multiple testing. Prevalence of presarcopenia and sarcopenia was low, but most prevalent in normal-weight NAFLD. However, in multivariable analysis neither sarcopenia nor presarcopenia was associated with NAFLD, most likely because of the relatively high SMI in this elderly community dwelling population. Second, high fat mass appeared a better predictor for NAFLD prevalence than low muscle mass. In particular, android fat mass had a significant association with higher NAFLD prevalence in all subgroups, whereas gynoid fat tended to be associated with lower NAFLD prevalence. Likewise, AGR as proxy for fat distribution was the strongest predictor for NAFLD.

Recently, low skeletal muscle mass emerged as potential risk factor for steatosis independent of age and BMI.[31–35] Most of these studies were carried out in Korean populations.[31–33,35] However, as ethnicity is important in the evaluation of body composition,[12] it is difficult to generalize these results to Western populations. Asian individuals carry proportionately higher fat mass for a given BMI than Caucasians. To date, only two studies have been performed in Western populations.[10,36] Both studies found that the prevalence of presarcopenia increased with fibrosis severity in NAFLD. But direct comparison to these studies is hampered by the different study populations (abovementioned studies included advanced steatohepatitis patients with cirrhosis prevalence of 33.3% and 15.6% without healthy control group).[10,36] This is in contrast to our study in which we targeted a different study population; ie, an unselected presumably healthy population in which only 10% of the participants with steatosis had coinciding elevated LSM. Furthermore, low skeletal muscle mass alone has been systematically referred to as sarcopenia in these previous reports. However, loss of strength or performance is strictly needed to make the actual diagnosis of sarcopenia.[15] A recent small study examined actual sarcopenia univariately with NAFLD and found that sarcopenia was actually associated with lower instead of higher NAFLD prevalence.[37] Yet, this finding is in line with our univariate results in which SMI was also associated with higher prevalence of NAFLD. Although the previous study did not perform a multivariable analysis, we found that this association changed after adjustment for weight.

Another commonly used proxy for skeletal muscle mass is ALM-fraction.[9,10,31–35] We chose to use SMI as advised by the EWGSOP consensus guidelines, but we did perform additional analyses on ALM-fraction, and found that both ALM fraction and SMI were equally good in predicting the prevalence of NAFLD if only adjusted for weight and height. We hypothesize that the explanation lies in the fact that most phenotypes associated with sarcopenia, are typically related to frailty and poor nutritional status (and hence to a low BMI),[15] whereas NAFLD is typically related to adiposity and overnutrition (and thus to a high BMI).[1] We would therefore like to pose that ALM-fraction might be more clinically applicable than SMI for the (univariate) assessment of presarcopenia in NAFLD as it already takes into account the confounder weight. In our study, neither presarcopenia nor sarcopenia was independently associated with NAFLD. We believe this is due to the low number of (pre)sarcopenic cases. Indeed, the median SMI was relatively high in our population. As a consequence, (pre) sarcopenia in the obese and overweight was rare (prevalence of 0.4% and 5%, respectively). This could explain the lack of SMI being associated with overweight NAFLD. As for muscle function, grip strength has been analyzed previously in a large Asian cohort that found an inverse association between grip strength (relative to body weight) and NAFLD.[38] In our study, incremental grip strength was associated with lower NAFLD prevalence in men only; however, this association did not hold significance after multivariable adjustment.

Interestingly, the association between SMI and NAFLD was more pronounced in women than in men. This is in contrast to previous studies in which associations are generally more pronounced in men. However, there were fewer men than women in this study (n = 1980 versus n = 2629) and, as said before, SMI was relatively high in our community-dwelling population. Therefore, we cannot fully exclude the possibility that differences in sex-stratified analyses resulted, in part, from power issues. Nevertheless, there are obvious differences in sex hormones between men and women that could have affected the association between SMI with NAFLD. As our study concerns an elderly population, there is a presumed relative reduced testosterone, a strong anabolic hormone, in men.[39] Nonetheless, absolute testosterone in elderly men is generally still higher than in women.[40] Also, relative estrogen is decreased in women because of the menopause,[39] and fat mass–to–fat free mass ratio is much higher in women than in men.[25] Furthermore, apart from the differences in sex hormones, other signaling hormones such as growth hormone could have also affected the relation between body composition and NAFLD.[1] In addition, inflammation has been suggested to play a major role in sarcopenia; myokines secreted by skeletal muscle mass, for instance, antagonize the pro-inflammatory and metabolic effects of adipocytes from fat tissue.[34,42] This could explain the hypothesized synergistic effect of low skeletal muscle mass and adiposity together. Moreover, it is known that visceral fat, in

particular, impairs adipocyte function and adipocytokine secretion, which can lead to an increase in pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-alpha.[43] This could explain the attenuating effect of AGR between body components with NAFLD, which is particularly interesting because AGR was much higher in men than in women.

A major strength of our study is the use of a large population-based cohort with access to a great number of reliably measured traits. Additionally, body composition was determined by the gold standard (DXA scan) and we used the recommended EWGSOP definition to determine the presence of presarcopenia and sarcopenia.[15] Moreover, we included only individuals with a European background to exclude bias due to racial differences in body composition. And last, we performed several sensitivity analyses to test the robustness of our conclusions. Nonetheless, several limitations need mentioning. First, the cross-sectional design of this study makes it impossible to draw conclusions on the cause-effect relation between body composition and NAFLD. Second, the gold standard for diagnosis of NAFLD is liver biopsy rather than US. However, performing an invasive liver biopsy in presumed healthy individuals is unethical. Moreover, US is a widely used screening tool that yields high sensitivity and specificity for moderate and severe steatosis.[44] Third, missing data on gait speed was substantial (32.6%). Yet, grip strength, as indicator for muscle strength, was present in almost all participants (>99%). Moreover, sensitivity analyses using imputed data on gait speed showed similar results to the main non-imputed analysis, suggesting no under or overestimation of sarcopenia. Fourth, we had no information on possible fat infiltration in the muscle, which could have affected the quality of muscle mass. Previous studies have suggested that, in older women particularly, there is quite a proportion of fat within the quadriceps muscle, this infiltration was related to gait speed.[45] However, in our study was gait speed not associated with NAFLD, neither in men nor in women.

In summary, skeletal muscle mass as assessed by multiple proxies was consistently associated with NAFLD in normal- weight women. However, fat mass was a better predictor for NAFLD probability in both sexes. In particular, android-fat-to- gynoid-fat ratio was strongly associated with NAFLD. This is in line with android fat being associated with higher odds and gynoid fat being associated with lower odds for NAFLD. These findings, if confirmed by others, add to the rationale of resistance training to replace fat mass by lean mass (regardless of of simultaneous weight loss) as an easily accessible, inexpensive, and targeted approach for individuals with NAFLD.[46]

Detailed acknowledgments and online resources can be found in the published article online

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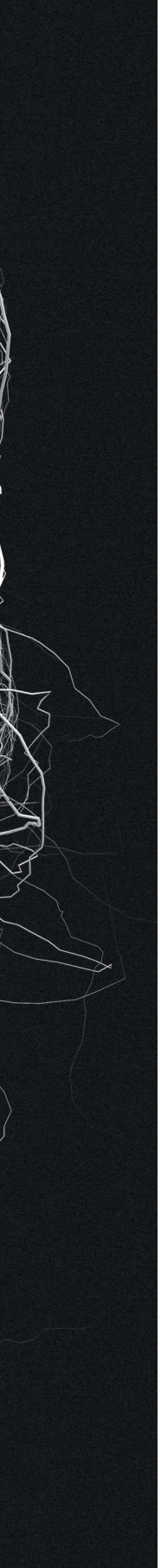
REFERENCES

1. Rinella ME.Nonalcoholic fatty liver disease: a systematic reviewJAMA.2015;313(22):2263–73.
2. Koehler EM, Schouten JN, Hansen BE, et al.Prevalence and risk factors of non-alcoholic fatty liver disease in the elderly: results from the Rotterdam study.J Hepatol.2012;57(6):1305–11.
3. Pais R, Barritt AS 4th, Calmus Y, et al.NAFLD and liver transplantation: current burden and expected challenges.J Hepatol.2016;65(6):1245–57.
4. Younossi ZM, Stepanova M, Negro F, et al.Nonalcoholic fatty liver disease in lean individuals in the United States.Medicine (Baltimore). 2012;91(6):319–27.
5. Prentice AM, Jebb SA.Beyond body mass index.Obes Rev.2001;2(3):141–7.
6. Pang Q, Zhang JY, Song SD, et al.Central obesity and nonalcoholic fatty liver disease risk after adjusting for body mass index.World J Gastroenterol. 2015;21(5):1650–62.
7. Ko YH, Wong TC, Hsu YY, Kuo KL, Yang SH.The correlation between body fat, visceral fat, and nonalcoholic fatty liver disease.Metab Syndr Relat Disord. 2017;15(6):304–11.
8. Wijarnpreecha K, Panjawatanan P, Thongprayoon C, Jaruvongvanich V, Ungprasert P.Sarcopenia and risk of nonalcoholic fatty liver disease: a meta-analysis.Saudi J Gastroenterol. 2018;24(1):12–7.
9. Koo BK, Kim D, Joo SK, et al.Sarcopenia is an independent risk factor for non-alcoholic steatohepatitis and significant fibrosis.J Hepatol.2017;66(1):123–31.
10. Petta S, Ciminnisi S, Di Marco V, et al.Sarcopenia is associated with severe liver fibrosis in patients with non-alcoholic fatty liver disease.Aliment Pharmacol Ther. 2017;45(4):510–8.
11. Bugianesi E, Gastaldelli A, Vanni E, et al.Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms.Diabetologia.2005;48(4):634–42.
12. Agrawal S, Duseja AK.Non-alcoholic fatty liver disease: east versus west.J Clin Exp Hepatol. 2012;2(2):122–34.
13. Lee DH, Keum N, Hu FB, et al.Predicted lean body mass, fat mass, and all cause and cause specific mortality in men: prospective US cohort study.BMJ.2018;362:k2575.
14. Ikram MA, Brusselle GGO, Murad SD, et al.The Rotterdam Study: 2018 update on objectives, design and main results.Eur J Epidemiol.2017;32(9):807–50.
15. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al.Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People.Age Ageing.2010;39(4):412–23.
16. Verlinden VJ, van der Geest JN, Hoogendam YY, Hofman A, Breteler MM, Ikram MA.Gait patterns in a community-dwelling population aged 50 years and older. Gait Posture.2013;37(4):500–5.
17. Trajanoska K, Schoufour JD, Darweesh SK, et al.Sarcopenia and its clinical correlates in the general population: The Rotterdam Study.J Bone Miner Res. 2018;33(7):1209–18.
18. Hamaguchi M, Kojima T, Itoh Y, et al.The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation.Am J Gastroenterol. 2007;102(12):2708–15.
19. Koehler EM, Plompen EP, Schouten JN, et al.Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam study.Hepatology.2016;63(1):138–47.

20. Boursier J, Zarski JP, de Ledinghen V, et al. Determination of reliability criteria for liver stiffness evaluation by transient elastography.Hepatology.2013;57(3):1182–91.
21. Petta S, Maida M, Macaluso FS, et al.The severity of steatosis influences liver stiffness measurement in patients with nonalcoholic fatty liver disease.Hepatology.2015;62(4):1101–10.
22. Goldbohm RA, van den Brandt PA, Brants HA, et al. Validation of a dietary questionnaire used in a large-scale prospective cohort study on diet and cancer. Eur J Clin Nutr. 1994;48(4):253–65.
23. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.Diabetologia.1985;28(7):412–9.
24. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity.Circulation.2009;120(16):1640–5.
25. Lafortuna CL, Maffiuletti NA, Agosti F, Sartorio A. Gender variations of body composition, muscle strength and power output in morbid obesity.Int J Obes (Lond). 2005;29(7):833–41.
26. Sakamoto Y, Ishiguro M, Kitagawa G Akaike information criterion statistics. Dordrecht, Netherlands: D. Reidel Publishing, 1986.
27. Hu FB, Stampfer MJ, Rimm E, et al.Dietary fat and coronary heart disease: a comparison of approaches for adjusting for total energy intake and modeling repeated dietary measurements.Am J Epidemiol. 1999;149(6):531–40.
28. Willett WNutritional epidemiology. New York: Oxford University Press, 2013.
29. Šidák Z. Rectangular confidence regions for the means of multivariate normal distributions.J Am Stat Assoc. 1967;62(318):626–33.
30. Galwey NW.A new measure of the effective number of tests, a practical tool for comparing families of non-independent significance tests.Genet Epidemiol.2009;33(7):559–68.
31. Hong HC, Hwang SY, Choi HY, et al. Relationship between sarcopenia and nonalcoholic fatty liver disease: the Korean Sarcopenic Obesity Study.Hepatology.2014;59(5):1772–8.
32. Lee YH, Jung KS, Kim SU, et al. Sarcopaenia is associated with NAFLD independently of obesity and insulin resistance: Nationwide surveys (KNHANES 2008-2011). J Hepatol.2015;63(2):486–93.
33. Kim HY, Kim CW, Park CH, et al.Low skeletal muscle mass is associated with non-alcoholic fatty liver disease in Korean adults: the Fifth Korea National Health and Nutrition Examination Survey.Hepatobiliary Pancreat Dis Int. 2016;15(1):39–47.
34. Moon JS, Yoon JS, Won KC, Lee HW.The role of skeletal muscle in development of nonalcoholic Fatty liver disease.Diabetes Metab J. 2013;37(4):278–85.
35. Kim G, Lee S-E, Lee Y-B, et al.Relationship between relative skeletal muscle mass and nonalcoholic fatty liver disease: a 7-year longitudinal study.Hepatology.2018;68(5):1755–68.
36. Issa D, Alkhouri N, Tsien C, et al.Presence of sarcopenia (muscle wasting) in patients with nonalcoholic steatohepatitis.Hepatology.2014;60(1):428–9.
37. Zhai Y, Xiao Q, Miao J. The relationship between NAFLD and sarcopenia in elderly patients.Can J Gastroenterol Hepatol. 2018;2018:5016091–4.

38. Meng G, Wu H, Fang L, et al. Relationship between grip strength and newly diagnosed nonalcoholic fatty liver disease in a large-scale adult population. Sci Rep. 2016;6:33255.
39. Sakuma K, Yamaguchi A. Sarcopenia and age-related endocrine function. Int J Endocrinol. 2012;2012:10–10.
40. Maggio M, Lauretani F, Ceda GP. Sex hormones and sarcopenia in older persons. Curr Opin Clin Nutr Metab Care. 2013;16(1):3–13.
41. Fan Y, Menon RK, Cohen P, et al. Liver-specific deletion of the growth hormone receptor reveals essential role of growth hormone signaling in hepatic lipid metabolism. J Biol Chem. 2009;284(30):19937–44.
42. Walsh K. Adipokines, myokines and cardiovascular disease. Circ J. 2009;73(1):13–8.
43. Schaffler A, Scholmerich J, Buchler C. Mechanisms of disease: adipocytokines and visceral adipose tissue—emerging role in nonalcoholic fatty liver disease. Nat Clin Pract Gastroenterol Hepatol. 2005;2(6):273–80.
44. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. J Hepatol. 2009;51(6):1061–7.
45. Visser M, Kritchevsky SB, Goodpaster BH, et al. Leg muscle mass and composition in relation to lower extremity performance in men and women aged 70 to 79: the health, aging and body composition study. J Am Geriatr Soc. 2002;50(5):897–904.
46. Hallsworth K, Fattakhova G, Hollingsworth KG, et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. Gut. 2011;60(9):1278–83.

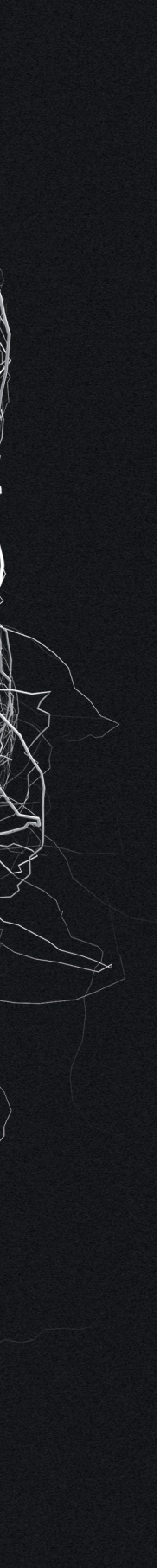




4

Genetic pleiotropy of the musculoskeletal system





4.1

Genetics of bone and muscle interactions in humans

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Curr Osteoporos Rep. *2019 Apr;17(2):86-95.*

ABSTRACT

Purpose of Review: To summarize the evidence from recent studies on the shared genetics between bone and muscle in humans.

Recent Findings: Genome-wide association studies (GWAS) have successfully identified a multitude of loci influencing the variability of different bone or muscle parameters, with multiple loci overlapping between the traits. In addition, joint analyses of multiple correlated musculoskeletal traits (i.e., multivariate GWAS) have underscored several genes with possible pleiotropic effects on both bone and muscle including *MEF2C* and *SREBF1*. Notably, several of the proposed pleiotropic genes have been validated using human cells or animal models.

Summary: It is clear that the study of pleiotropy may provide novel insights into disease pathophysiology potentially leading to the identification of new treatment strategies that simultaneously prevent or treat both osteoporosis and sarcopenia. However, the role of muscle factors (myokines) that stimulate bone metabolism, as well as osteokines that affect muscles, is in its earliest stage of understanding.

INTRODUCTION

Osteoporosis and sarcopenia are common and costly comorbid diseases of aging, and there is an urgent need to prevent and treat both to reduce their associated morbidity and mortality.[1**] A burgeoning body of work shows that they share many common risk factors and biological pathways such as effects of growth hormone and inflammatory cytokines.[2,3] Moreover, the common mesenchymal origin of bone and muscle cells underpins the tight link between these conditions from the early stages of embryonic development.

Muscle mass and function are important determinants of skeletal growth and bone mass accrual in growing humans. This adaption of bone tissue to loading follows the principles of Frost's mechanostat theory, i.e., bone growth and bone loss are stimulated by the muscle forces/loads acting upon bone surfaces.[4] In addition to their mechanical interaction, bone and muscle are jointly regulated by hormones, and inextricably linked genetically and molecularly.[5] However, the latter interactions are difficult to observe and measure; thus, their roles are less well recognized. The recent rise of new technologies (arising from genetics, molecular biology etc.) has shed new light on the genetic and molecular interplay between bone and muscle; thus, our understanding of these interactions has evolved over time. In the past few years, studies have endeavored to (1) disentangle the intricate molecular mechanisms that lead to osteoporosis and sarcopenia and (2) develop new treatment strategies by pinpointing drug targets common to both conditions.

The aim of this review is to summarize the evidence from current studies on the shared genetics between bone and muscle in humans. Another recent review has addressed this topic in mouse models.[6]

GENOME-WIDE ASSOCIATION STUDY FOR BONE OR MUSCLE-RELATED PHENOTYPES

Risk factors affecting osteoporosis and sarcopenia have a strong genetic component, with heritability estimates above 60%.[7] Genome-wide association studies (GWAS) have identified multiple genetic variants influencing the variability of bone mass (**Figure 1**). In total, 62 loci[8-12] have been associated with DXA-derived bone mineral density (BMD) at either the femoral neck or lumbar spine, while 36 loci[13] have been associated with total body BMD. Notably, these GWASs have highlighted known bone-active pathways, i.e., OPG-RANK-RANKL, WNT, and mesenchymal differentiation, among others.[14] One of the greatest successes in the osteoporosis field was achieved through the discovery of the BMD locus harboring *WNT16*, a critical regulator of cor-

tical bone thickness[15] and trabecular bone mass.[16] Moreover, with an ever-growing number of genes discovered by GWASs, novel pathways acting on bone have been identified (e.g., oncogenic and melanogenesis pathways). Recently, 518 loci have been associated with ultrasound-derived heel BMD,[17,18] estimated in more than 400,000 participants of the UK Biobank (UKBB) study. Together, these studies have provided new insights into the pathophysiology of osteoporosis, illustrated by the discovery and functional validation of *GPC6* and *DAAM2*. *GPC6* may serve as novel drug target for osteoporosis, since it encodes glypican, which is involved in cellular growth control and differentiation. Moreover, GPC6 loss of function leads to increased bone mineral content and developmental skeletal abnormalities. *DAAM2* also may be a potential drug target for osteoporosis as it shows effects on bone strength, porosity, and quality in murine models by indirect regulation of the canonical Wnt signaling. *DAAM2* was also expressed in human skeletal muscle (**Table1**).[19]

Table 1 | Bone genes discovered by UK Biobank (and other GWAS0 and evidence of their molecular role in the muscle

eBMD gene	Muscle-related trait	Reference
AHNAK	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
AQP1	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
ARHGAP26	Positive/mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018
BCKDHB	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
DAAM2	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
DLEU1	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
GRB10	Negative/mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018
HMGA2	Positive/mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018
IGFBP2	Negative/mouse skeletal muscle mass overexpress	Verbrugge. Schönfelder et al. 2018
MMP9	Positive/mouse skeletal muscle mass overexpress	Verbrugge. Schönfelder et al. 2018
MPP7	Gene expression in human skeletal muscle	Su. Ekman et al. 2015
PPARD	Positive/mouse skeletal muscle mass overexpress	Verbrugge. Schönfelder et al. 2018
SMAD3	Positive/ mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018
SMAD7	Positive/mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018
SOX6	Positive/mouse skeletal muscle mass KO	Verbrugge. Schönfelder et al. 2018

eBMD= estimated bone mineral density, KO knock out

In contrast, the fewer number of GWAS of muscle-related phenotype provide less biological insight about the pathways leading to the development of sarcopenia (**Figure 1**). To date, only five loci (*HSD17B11, VCAN, ADAMTSL3, IRS1*, and *FTO*) have been robustly associated with lean mass (total and/ or appendicular),[20] which constitutes a good proxy for skeletal muscle mass.[21] Three out of the five lean mass-associated SNPs identified by GWAS are significantly enriched in enhancers and promoters acting in

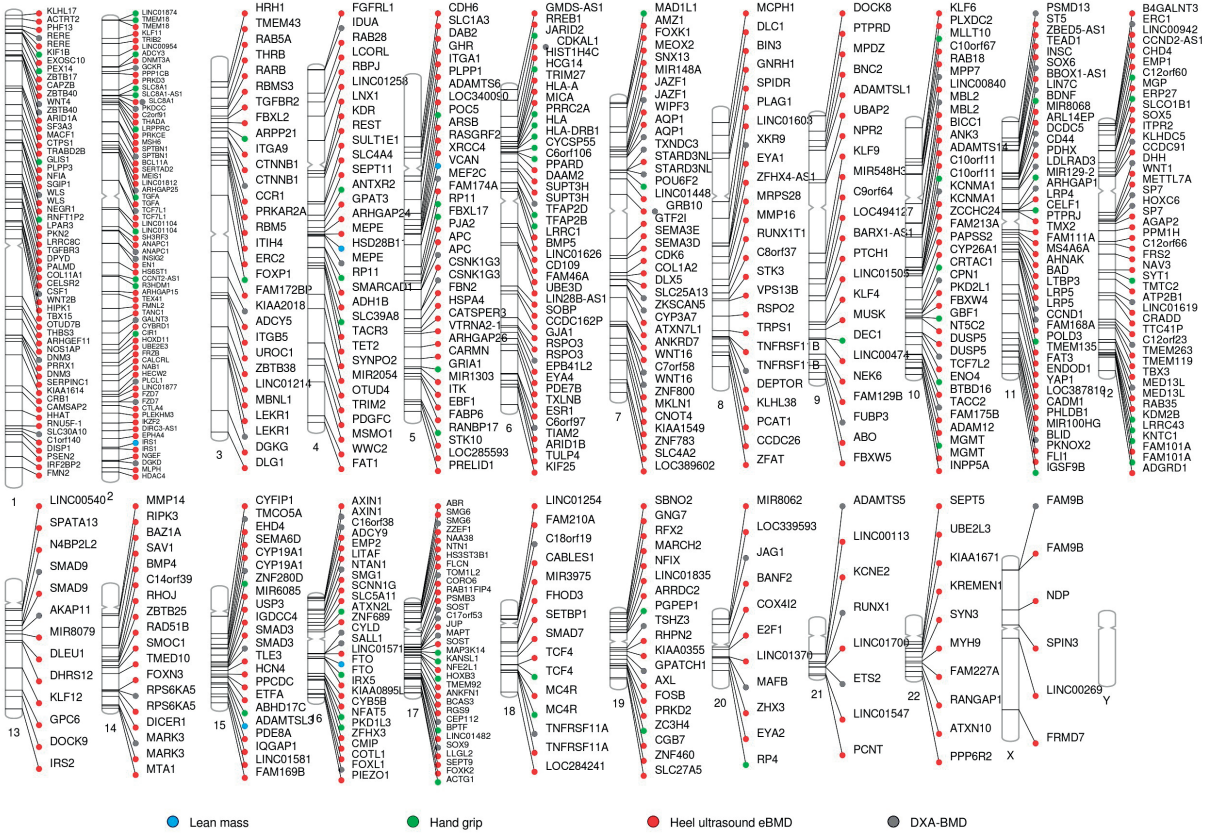


Figure 1 | Phenogram showing genome-wide association study results for bone and muscle-related phenotypes. Genes mapping to loci associated with lean mass (light blue), hand grip (light green), heel ultrasound estimated BMD (red), and DXA-derived BMD (gray). The ideogram was constructed using Phenogramhttp://visualization.ritchielab.psu.edu/phenograms/plot

muscle cells. Recently, the same study identified *TNRV6B* as additional lean mass locus after more stringent adjustment for fat.[22] However, the exact biological pathways affecting muscle mass still remain unknown. Two recent grip strength GWAS, a proxy for muscular function, have been more fruit-ful, yielding 64 muscle strength-related loci[23,24] identified within the UK Biobank. The loci found associated with grip strength contain genes implicated in the structure and function of skeletal muscle (*ACTG1*), excitation-contraction coupling (*SLC8A1*), or involvement in the regulation of neurotransmission (*SYT1*), which provides additional evidence of the genetic control exerted on this muscle trait.[23] These findings higlight that the grip strength phenotype has a neuromuscular component, since it also characterizes the ability of the peripheral nervous system to appropriately recruit muscle cells. Briefly, Actg1-ms knockout mice display muscle weakness and whole-body functional deficit. [25] *SLC8A1* overexpression in muscle cells induces muscular changes similar to those of muscular dystrophy.[26]

Finally, *SYT1* has been linked to synaptic defects at the neuromuscular junctions in mouse model of spinal muscular atrophy.[27] Further, three lead SNPs (rs10186876, rs6687430, and rs754512) for grip strength map in the vicinity of genes implicated in monogenic syndromes characterized by neurological and/or psychomotor impairment like French-Canadian variant of Leigh syndrome characterized *(LRPPRC*), Zellweger Spectrum Peroxisomal Biogenesis Disorder (*PEX14*), and Koolen-de Vries syndrome (*KANSL1*).[23]

Table 2 | Overlapping genes between different bone parameters and different muscle-related traits

Muscle-related trait	Gene	eBMD *P* value	Muscle-related traits *P* value	Reference
Bone mineral density				
Total body lean mass	*MC4R*	2.0×10^{-15}	1.0×10^{-18}	Karasik et al. 2019
Total body lean mass	*FTO*	1.6×10^{-26}	1.4×10^{-09}	Zillikens et al. 2017
Hand grip strength	*IRS1*	4.7×10^{-08}	1.5×10^{-11}	Zillikens et al. 2017
Hand grip strength	*MGMT*	2.3×10^{-22}	1.0×10^{-13}	Tikkanen et al. 2018
Hand grip strength	*TCF4*	9.4×10^{-10}	5.9×10^{-15}	Tikkanen et al. 2018
Hand grip strength	*TMEM18*	2.0×10^{-11}	5.4×10^{-22}	Tikkanen et al. 2018
Hand grip strength	*LINC01104*	7.9×10^{-11}	3.1×10^{-09}	Tikkanen et al. 2018
Hand grip strength	*MC4R*	2.0×10^{-15}	2.1×10^{-19}	Tikkanen et al. 2018
Hand grip strength	*PEX14*	6.7×10^{-13}	5.6×10^{-11}	Willems et al. 2017
Hand grip strength	*SLC8A1*	7.4×10^{-38}	7.7×10^{-09}	Willems et al. 2017
Hand grip strength	*TGFA*	9.3×10^{-19}	4.8×10^{-13}	Willems et al. 2017
Bivariate analysis with bone strength index				
Appendicular lean mass	*FADS1*		$p_b = 1.6 \times 10^{-07}$	Han et al. 2012
Bivariate analysis with appendicular bone size				
Appendicular lean mass	*GLYAT*		$p_b = 1.8 \times 10^{-06}$	Guo et al. 2013

pb bivariate p value for bone/muscle pair, eBMD estimated bone mineral density.

FROM CROSS-PHENOTYPE EFFECTS TO PLEIOTROPY: BONE AND MUSCLE

Basic Concepts

Multiple genes identified by GWAS of muscle-related traits have also been associated with heel BMD in the UKBB GWAS **(Table 2**). While such cross-phenotype associations may arise due to biological pleiotropy, there are other reasons that can lead to spurious pleiotropy. Therefore, cross-phenotype associations should not be always regarded as the consequence of true pleiotropy. Pleiotropy commonly refers to a phenomenon in which a genetic locus (a gene or a single variant within a gene) affects more than one trait or disease.[28] It can be classified as (1) biological-when a gene has a direct biological effect on more than one trait or biomarker; (2) mediated-where a gene has a biological effect on one trait which lies on the causal path to another trait and thus the gene affects both traits; and (3) spurious-when different forms of biases can lead to false-positive findings.[29] The most common causes of spurious pleiotropy are ascertainment bias and phenotypic misclassification.[29] The study of pleiotropy may have tremendous clinical implications in the fields of osteoporosis and sarcopenia by discovering new drug targets acting on both muscle and bone.

Shared biology: Evidence from Multivariate Analysis

While GWAS are typically performed for the study of one trait at a time, more recently methodological advances have enabled the simultaneous GWAS assessment of multiple traits. In humans, joint analysis of multiple, correlated traits, i.e., multivariate GWAS, has been instrumental to the identification of pleiotropic candidate SNPs/loci associated with traits related to both bone and muscle metabolism. GWAS investigating both bone and muscle phenotypes have produced a list of potential candidate genes for further biological validation such as *PRKCH* and *SCNN1B*[30] in 3844 Europeans; *HK2*, *UMOD*, *MIR873*, and *MIR876*[31] in 1627 unrelated Chinese adults individuals; *HTR1E, COL4A2, AKAP6, SLC2A11, RYR3*, and *MEF2C;*[32] and *GLYAT*[33] in 1627 unrelated Chinese adults. In addition, the GWAS-identified *METTL21C* was found to be a novel pleiotropic gene suggestive of asso-ciation with both muscle and bone acting through the modulation of the NF-κB signaling pathway.[34**] This gene has been implicated in the etiology of inclusion body myositis (skeletal muscle) and of early-onset Paget's disease (bone).[35] Subsequent studies confirmed that *METTL21C* polymorphisms contribute to peak bone mass in Chinese males.[36] Moreover, frail subjects showed higher expression levels of METTl21C compared to young healthy older adults.[37] *METTL21C* belongs to the METTL21 family of the methyl-transferase superfamily and possesses protein-lysine N-meth-yltransferase activity; [38] its close homolog, METTL21D was found to bind to the chaperone valosin-containing protein (VCP, a.k.a. VCP/p97),

known to play a role in a muscle atrophy disease.[39] More recently, Medina-Gomez et al. [40**] performed a bivariate GWAS meta-analysis of total body lean mass and total body less head BMD in 10,414 children. The study identified variants with pleiotropic effects in eight loci, mostly already known for BMD (*WNT4, GALNT3, MEPE, CPED1/WNT16, TNFSF11, RIN3*, and *PPP6R3/LRP5*), but also the *TOM1L2/SREBF1* locus not previously associated with BMD or lean mass. The protein was highly expressed in mouse calvaria-derived cells during osteoblastogenesis and showed the highest expression peak at the onset of osteoblast mineralization in human mesenchymal stem cells. Moreover, *SREBP1* indirectly downregulated several key regulators of myogenesis (i.e., MYOD1, MYOG, MEF2C). Notably, *SREBF1* exerted opposite effects on the differentiation of myocytes and osteoblasts, which are in line with the opposite effects observed on BMD and lean mass in the bivariate analysis.

HUMAN AND ANIMAL BONE-FOCUSED KNOCK-OUT "MODELS" COMPRISING MUSCLE PHENOTYPES

Congenital disorders affecting bone or muscle are often associated with deficits in the other tissue as well. For example, reduced muscle capacity and strength have been observed in children with osteogenesis imperfecta (OI), [41,43] where the primary defect comprises the skeletal system. About 85% of the OI cases are caused by mutations in the *COL1A1* and *COL1A2* genes. These mutations affect the production of the α1/α2 chains of type 1 collagen, an important structural component of the bone, skin, tendons, ligaments, and other connective tissues.[44] Animal studies have also observed muscle weakness in OI mice,[45] providing additional evidence for the muscle abnormalities in OI. The exact mechanisms leading to muscle weakness are yet unclear but they can be result of intrinsic muscle factors or direct paracrine effects of the abnormal bone matrix (i.e., increased TGF-β signaling in OI decreases lean mass). Further, muscle abnormalities have been also noted in individuals with hypophosphatemic rickets; hereditary phosphate wasting disorders commonly caused by point mutations in *PHEX, FGF23,* and *DMP1* genes. Children carriers of any of these mutations have soft bones (rickets), growth retardation, poor dental development, and elevated serum FGF23 levels.[46] It has been shown that accumulation of *FGF23* is strongly associated with muscle function abnormalities in humans. [47,48] Additionally, murine models have shown reduced grip strength and impaired muscle forces in the Hyp (model of PHEX deficiency) and Dmp1 null (DMP1 deficiency) mice.[49] However, the underlying mechanisms leading to skeletal muscle abnormalities in individuals with hypophosphatemic rickets have not been characterized.

HUMAN AND ANIMAL KNOCK-OUT MODELS OF MUSCLE COMPRISING BONE PHENOTYPES

Disorders of muscle often present with bone abnormalities. For example, in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy, the primary defect leading to disease pathogenesis is degeneration of striated muscle. The mutations in the DMD gene encode the dystrophin protein, causing Duchenne and Becker muscular dystrophies. Yet, impairment of bone health in the form of low BMD and increased incidence of bone fractures are well-recognized clinical components of the DMD phenotype.[50-52] The deleterious effects of DMD on bone can be mediated by several mechanisms. These include downstream functional effects on bone involving the nuclear factor of κB pathway and cytokine-mediated (IL-6) activation of osteopontin (OPN); disturbances of calcium metabolism; deterioration of biomechanical stimuli with disease progression, side effects of corticosteroid treatment and many comorbidity processes derived from the disease. Also, other single-gene disorders including those derived from mutations in *MTM1, RYR1,* and *DNM2* have been implicated in the alteration of skeletal muscles. These congenital myopathies are characterized by an alteration in the contractile apparatus of the muscle (myofibers) followed by loss of muscle mass, increased muscle weakness, and decrease in bone mass. However, due to the complex interplay between muscle and bone, it is not clear if these changes in bone mass are result of the decreased mechanical loading, the muscular paracrine effect, the shared biology, or combination of all of these factors.

Another example of a potentially pleiotropic gene is the myostatin (*MSTN*, a.k.a. growth and differentiation factor 8, *GDF8*) gene, a member of the TGF-β superfamily, which is secreted primarily by the skeletal muscle cells.[53] Point mutations in *MSTN* lead to decreased production of functional myostatin causing muscle hypertrophy in humans and animals.[54] Recently, the relationship between myostatin and bone has been actively investigated in animal models. *MSTN* depletion leads to increased BMD and strength through many different pathways. First and foremost, the loss of myostatin is followed by doubling of the skeletal muscle mass, which in-creases mechanical loading on bone. However, the increase in lean mass can also have indirect positive effects on the bone by increasing IGF-1 levels.[55] Such studies have found that inhibition of the myostatin pathway increases proliferation and differentiation of osteoprogenitor cells and leads to bone mass accrual.[56] Further, it has been shown that haploinsufficiency of myostatin protects against aging-related declines in muscle function and enhances the longevity of mice.[57] This suggests that, beyond a known effect of myostatin on bone,[58] there is also a systemic effect of this gene.

Further, myostatin binds to the soluble activin type IIB receptor (ACVR2B) which forms an activin receptor complex with activin type 1 serine/threonine kinase

receptors (ACVRs). Loss of function of activin type I receptor (*ACVR1*) in osteoblasts increases bone mass and activates canonical Wnt signaling through suppression of the Wnt inhibitors SOST and DKK1.[59**] Recently, a study has also shown that myostatin inhibits osteoblastic differentiation by suppressing osteocyte-derived exosomal microRNA-218, suggesting a possible novel mechanism in the bone-muscle crosstalk. [60]

Myocyte enhancer factor-2 (MEF2C) is a member of the MADS-box superfamily of transcriptional regulatory proteins relevant for skeletal muscle development, sarcomeric gene expression, and fiber type control. MEF2C directly regulates myomesin gene transcription and loss of Mef2c in skeletal muscle results in improper sarcomere organization and disorganized myofibers. Recent studies have found that a super activating form of MEF2C causes precocious chondrocyte hypertrophy, ossification of growth plates, and dwarfism. [61] Mef2c+/− presented lack of ossification within the sternum. Mef2cloxP/KO; Twist2-Cre mice had shortened limbs from birth.[61] Moreover, the *MEF2* activity is enhanced by the increase in mitogen-activated protein kinases (MAPKs) and decrease in histone deacetylases (HDACs). HDAC inhibitors have been tested in a muscular dystrophy model in mice which promoted the formation of muscles with increased cross-sectional area. [62] Interestingly, HDAC5 part of the HDAC family is a known BMD locus. [8] Last but not least, GWAS studies have identified variants in *FAM210A* as strongly associated with fracture risk but less strongly with BMD. Moreover, SNPs near *FAM210A* were nominally ($p < 0.05$) associated with lean mass in adults. [1] Interestingly, a recent study in mice has shown that Fam210a was expressed in muscle mitochondria and cytoplasm but not in bone. [1] Notably, grip strength and limb lean mass were reduced in both tamoxifen-inducible Fam210a homozygous global knockout mice (TFam210a-/-) and skeletal muscle cell-specific knockout mice (Fam210aMus-/-). Moreover, microarray analysis showed decreased levels of Myog and Chdh15, transcription factors relevant for myoblast differentiation, and terminal muscle differentiation, respectively. Also, decreased BMD, bone biomechanical strength and bone for-mation, and elevated osteoclast activity were observed in TFam210a-/- mice. [2] Furthermore, the authors showed that Mmp12 was increased in muscle cells of TFam210aMus -/- mice, which can enhance osteoclast function in bone. Therefore, Fam210a, while being expressed in muscle, plays an influential role on bone quality and quantity.

MUSCLE AND BONE: BEYOND BIOMECHANICS

Multiple metabolic communications have been identified between bone and muscle in humans and rodents. There are numerous indications that the muscle "secretome" contains osteoinducer and osteoinhibitor myokines; [63] it also seems likely that bone

cells secrete myoinducer and myoinhibitor osteokines. [64] The skeletal muscle secretome accounts for various molecules that affect bone including insulin-like growth factor-1 (IGF1), fibroblast growth factor (FGF2), interleukins (IL6, IL15), myostatin, osteoglycin, osteoactivin, and others (reviewed by [63]). Even though studies on the potential effects of bone on muscle metabolism are still sparse, a few osteokines have been identified. Prostaglandin E2 (*PGE2*) and WNT family member 3A (*WNT3A*), which are secreted by osteocytes, are thought to impact skeletal muscle cells. Interestingly, WNT3A and several other WNT factors have been identified in GWAS of BMD. Also, osteocalcin and IGF-1, which are produced by osteoblasts, and sclerostin, which is secreted by osteocytes and osteoblasts, exert effects on muscle cells. Further, bones and muscles are controlled by mitochondrial genetics that standard GWAS cannot reliably scrutinize given the sparse number of mitochondrial markers on genotyping arrays and the difficulties to quantifying mitochondrial heteroplasmy. Previous studies have revealed the critical role of mitochondria in the differentiation of multiple cell types, including cardiomyocytes [65] and myoblasts.[66] Additionally, osteoporosis and sarcopenia seem to be more prevalent among patients with mitochondrial disorders. Thus, refined GWAS efforts can help understand the underlying mechanisms of the mitochondria, using intensity-based assessments of mitochondrial copy number. In addition to the mitochondrial metabolism, the genes controlling overall energy metabolism might also exert an impact on bone and muscle. Interestingly, skeletal muscle-specific disruption of the circadian rhythms by Bmal1 deletion has been shown to disrupt skeletal muscle metabolism, [67] whereas BMAL1 deficiency results in a low bone mass phenotype. [68] However, this gene has not yet been identified by any GWAS on BMD.

THE POTENTIAL OF GENETIC DISCOVERIES TO GUIDE DRUG TARGET IDENTIFICATION

Incorporating genetic information in the drug discovery process can improve disease-specific drug target identification and validation. Combining drug mechanisms with genetic mechanistic information increases the success in drug discovery over approaches that do not include genetic information, especially for drug targets related to musculoskeletal (BMD), metabolic, and blood traits. [69] From the molecular factors discussed above, two have been followed as potential drug targets. It has been well established that myostatin is a negative regulator of muscle and bone mass. Therefore, there is hope that studies into myostatin inhibitors may have therapeutic application in treating muscle-wasting diseases such as muscular dystrophy. Bialek et al. [58] have investigated the role of myostatin by administrating myostatin neutralizing antibody

(Mstn-mAb) or soluble myostatin decoy receptor (ActRIIB-Fc) in young adult mice. Interestingly, while both antibodies increased muscle mass, only ActRIIB-Fc also increased bone mass. Thus, a therapeutic agent that has this dual effect represents a potential approach for the simultaneous treatment of osteoporosis and or sarcopenia. Another potential target is FGF23. Currently, clinical trials of neutralizing anti-FGF23 antibody for patients with FGF23-related hypophosphatemic diseases are ongoing. First of all, FGF23 production is stimulated through signaling acting through the FGF receptor. It has also been shown that repeated administration of FGF receptor inhibitors causes increased bone growth and mineralization in Hyp mice. [70] Similarly, weekly injection of FGF23 antibodies increased BMD in Hyp mouse, while with a higher dose, there was also an increase in grip strength. To note, in a phase I clinical trial, administration of various amounts of anti-FGF23 antibodies increased tubular maximum transport of phosphate per glomerular filtration rate (TmP/GFR) in adult patients with X-linked hypophosphatemia (XLH). [71,72] Nevertheless, it needs to be tested if anti-FGF23 antibody can improve or cure rickets/osteomalacia or their clinical presentations such as bone pain and muscle weakness. Although the clinical implications of these findings are still farreaching, both examples illustrate the diverse opportunities for the characterization of drug targets that can prevent muscle and bone abnormalities. The approach is not free of limitations, as genetically derived targets may also have undesired secondary effects. For example, *MEF2C* has been suggested as novel drug target for therapeutically enhancing muscle performance. Targeting these genes may have a significant impact on the treatment of muscle. However, *MEF2C* also is related with pathological cardiac hypertrophy. Hence, off-target effects can be considerable. The key will be to improve muscle performance and prevent cardiotoxicity at the same time when targeting these genes. [73] Either way, this also illustrates the potential of the genetic evidence underlying drug targets to typify the presence of adverse effects before embarking on expensive experimentation.

SUMMARY AND FUTURE DIRECTIONS

Overall, there are many evolutionary, biological, and clinical factors that couple the pathogenesis of sarcopenia and osteoporosis. Both muscle and bone also act as endocrine organs [74] and share common genetic influences. Although challenging, there is a growing research enterprise aimed at elucidating and unraveling new mechanisms of muscle-bone crosstalk. Many questions still remain unanswered and need to be addressed through the integration of in vitro and in vivo models. For example, what are the exact mechanisms underlying the cross-organ effects? Do muscle factors, by stimulating bone metabolism, also lead to increased release of myoinducer and

myoinhibitor osteokines? More importantly, the question remains as how the aging process influences muscle and bone metabolism, including the underlying molecular factors. Further, the role of central mechanisms in co-regulation of the musculoskeletal system needs to be investigated in its entirety rather than its parts. Finally, the study of pleiotropy may provide novel insights into disease pathophysiology with the potential of leading to the identification of drug targets that simultaneously prevent or treat both diseases.

REFERENCES

Reference Papers of particular interest, published recently, have been highlighted as:
•• Of major importance

1. •• Tanaka K-I, Xue Y, Nguyen-Yamamoto L, Morris JA, Kanazawa I, Sugimoto T, et al. FAM210A is a novel determinant of bone and muscle structure and strength. Proc Natl Acad Sci U S A. 2018;115:E3759–68. This study have succesfully validated FAM210A as novel gene associated with reduced bone mass and grip strength in genetically modifed mice. FAM210A had been previously discovered to be associated with BMD using GWAS approach.
2. Reginster J-Y, Beaudart C, Buckinx F, Bruyère O. Osteoporosis and sarcopenia. Curr Opin Clin Nutr Metab Care. 2016;19:31–6.
3. Edwards MH, Dennison EM, Aihie Sayer A, Fielding R, Cooper C. Osteoporosis and sarcopenia in older age. Bone. 2015;80:126– 30.
4. Frost HM. Bone's mechanostat: a 2003 update. Anat Rec A Discov Mol Cell Evol Biol. 2003;275:1081–101.
5. Avin KG, Bloomfield SA, Gross TS, Warden SJ. Biomechanical aspects of the muscle-bone interaction. Curr Osteoporos Rep. 2015;13:1–8.
6. Verbrugge SAJ, Schönfelder M, Becker L, Yaghoob Nezhad F, Hrabě de Angelis M, Wackerhage H. Genes Whose Gain or Loss-Of-Function Increases Skeletal Muscle Mass in Mice: A Systematic Literature Review. Front Physiol. 2018;9:9.
7. Karasik D, Kiel DP. Genetics of the musculoskeletal system: a pleiotropic approach. J Bone Miner Res. 2008;23:788–802.
8. Estrada K, Styrkarsdottir U, Evangelou E, Hsu Y-H, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012;44:491–501.
9. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011;7:e1001372.
10. Rivadeneira F, Styrkársdottir U, Estrada K, Halldórsson BV, Hsu Y-H, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009;41:1199–206.
11. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008;358:2355–65.
12. Richards J, Rivadeneira F, Inouye M, Pastinen T, Soranzo N, Wilson S, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008;371:1505–12.
13. Medina-Gomez C, Kemp JP, Trajanoska K, Luan J, Chesi A, Ahluwalia TS, et al. Life-course genome-wide association study meta-analysis of Total body BMD and assessment of age-specific effects. Am J Hum Genet. 2018;102:88–102.
14. Rivadeneira F, Mäkitie O. Osteoporosis and bone mass disorders: from gene pathways to treatments. Trends Endocrinol Metab. 2016;27:262–81.

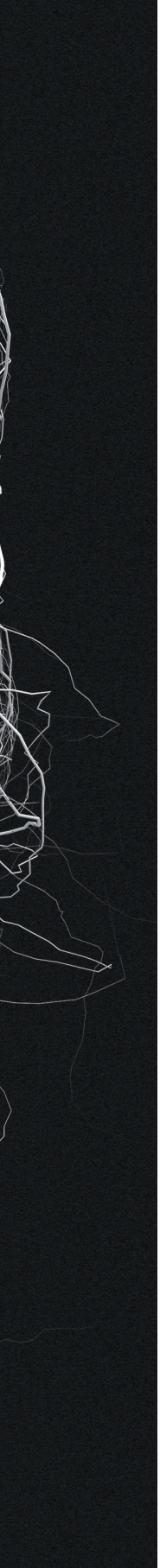
15. Ohlsson C, Henning P, Nilsson KH, Wu J, Gustafsson KL, Sjögren K, et al. Inducible Wnt16 inactivation: WNT16 regulates cortical bone thickness in adult mice. J Endocrinol. 2018;237:113–22.
16. Movérare-Skrtic S, Wu J, Henning P, Gustafsson KL, Sjögren K, Windahl SH, et al. The bone-sparing effects of estrogen and WNT16 are independent of each other. Proc Natl Acad Sci U S A. 2015;112:14972–7.
17. Kemp JP, Morris JA, Medina-Gomez C, Forgetta V, Warrington NM, Youlten SE, et al. Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis. Nat Genet. 2017;49:1468–75.
18. Morris JA, et al. An atlas of genetic influences on osteoporosis in humans and mice. Nat. Genet. 2018.
19. Su J, Ekman C, Oskolkov N, Lahti L, Ström K, Brazma A, et al. A novel atlas of gene expression in human skeletal muscle reveals molecular changes associated with aging. Skelet Muscle. 2015;5: 35.
20. Zillikens MC, Demissie S, Hsu Y-H, Yerges-Armstrong LM, Chou W-C, Stolk L, et al. Large meta-analysis of genome-wide association studies identifies five loci for lean body mass. Nat Commun. 2017;8:80.
21. Chen Z, Wang Z, Lohman T, Heymsfield SB, Outwater E, Nicholas JS, et al. Dual-energy X-ray absorptiometry is a valid tool for assessing skeletal muscle mass in older women. J Nutr. 2007;137:2775–80.
22. Karasik D, Zillikens MC, Hsu YH, Aghdassi A, Akesson K, Amin N, et al. Disentangling the genetics of lean mass. Am J Clin Nutr. 2019;109:276–287.
23. Willems SM, Wright DJ, Day FR, Trajanoska K, Joshi PK, Morris JA, et al. Large-scale GWAS identifies multiple loci for hand grip strength providing biological insights into muscular fitness. Nat Commun. 2017;8:16015.
24. Tikkanen E, Gustafsson S, Amar D, Shcherbina A, Waggott D, Ashley EA, et al. Biological insights into muscular strength: genetic findings in the UK biobank. Sci Rep. 2018;8:6451. https:// doi.org/10.1038/s41598-018-24735-y.
25. Sonnemann KJ, Fitzsimons DP, Patel JR, Liu Y, Schneider MF, Moss RL, et al. Cytoplasmic γ-actin is not required for skeletal muscle development but its absence leads to a progressive myopathy. Dev Cell. 2006;11:387–97. https://doi.org/10.1016/j.devcel.2006.07.001.
26. Burr AR, Millay DP, Goonasekera SA, Park KH, Sargent MA, Collins J, et al. Na+ dysregulation coupled with Ca2+ entry through NCX1 promotes muscular dystrophy in mice. Mol Cell Biol. 2014;34:1991– 2002.
27. Dale JM, Shen H, Barry DM, Garcia VB, Rose FF, Lorson CL, et al. The spinal muscular atrophy mouse model, SMAΔ7, displays altered axonal transport without global neurofilament alterations. Acta Neuropathol. 2011;122:331–41.
28. Paaby AB, Rockman MV. The many faces of pleiotropy. Trends Genet. 2013;29:66–73.
29. Solovieff N, Cotsapas C, Lee PH, Purcell SM, Smoller JW. Pleiotropy in complex traits: challenges and strategies. Nat Rev Genet. 2013;14:483–95.
30. Gupta M, Cheung C-L, Hsu Y-H, Demissie S, Cupples LA, Kiel DP, et al. Identification of homogeneous genetic architecture of multiple genetically correlated traits by block clustering of genome-wide associations. J Bone Miner Res. 2011;26:1261–71.
31. Sun L, Tan L-J, Lei S-F, Chen X-D, Li X, Pan R, et al. Bivariate genome-wide association analyses of femoral neck bone geometry and appendicular lean mass. PLoS One. 2011;6:e27325.

32. Karasik D, Cheung CL, Zhou Y, Cupples LA, Kiel DP, Demissie S. Genome-wide association of an integrated osteoporosis-related phenotype: is there evidence for pleiotropic genes? J Bone Miner Res. 2012;27:319–30.
33. Guo Y-F, Zhang L-S, Liu Y-J, Hu H-G, Li J, Tian Q, et al. Suggestion of GLYAT gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. Hum Genet. 2013;132:189–99.
34. •• Huang J, Hsu Y-H, Mo C, Abreu E, Kiel DP, Bonewald LF, et al. METTL21C Is a Potential Pleiotropic Gene for Osteoporosis and Sarcopenia Acting Through the Modulation of the NF-κB Signaling Pathway. J Bone Miner Res. 2014;29:1531–40. This study showed that METTL21C plays important role in mygenesis and osteocyte homeostasis via the NFκB signaling pathway using C2C12 myoblasts/ myotubes and MLO-Y4 osteocyte-like cells.
35. Cloutier P, Lavallée-Adam M, Faubert D, Blanchette M, Coulombe B. A newly uncovered Group of Distantly Related Lysine Methyltransferases Preferentially Interact with molecular chaperones to regulate their activity. PLoS Genet. 2013;9: e1003210.
36. Zhao F, Gao L, Li S, Wei Z, Fu W, He J, et al. Association between SNPs and haplotypes in the METTL21C gene and peak bone mineral density and body composition in Chinese male nuclear families. J Bone Miner Metab. 2017;35:437–47.
37. Hangelbroek RWJ, Fazelzadeh P, Tieland M, Boekschoten MV, Hooiveld GJEJ, van Duynhoven JPM, et al. Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness. J Cachexia Sarcopenia Muscle. 2016;7: 604–14.
38. Kernstock S, Davydova E, Jakobsson M, Moen A, Pettersen S, Mælandsmo GM, et al. Lysine methylation of VCP by a member of a novel human protein methyltransferase family. Nat Commun. 2012;3:1038.
39. Cohen S, Nathan JA, Goldberg AL. Muscle wasting in disease: molecular mechanisms and promising therapies. Nat Rev Drug Discov. 2015;14:58–74.
40. •• Medina-Gomez C, Kemp JP, Dimou NL, Kreiner E, Chesi A, Zemel BS, et al. Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/ TOM1L2 locus. Nat Commun. 2017;8:121. Using a bivaraite GWAS approach, this study have found a novel gene with pleiotropic effects on bone and muscle, which was expressed in murine and human osteoblasts, as well as in human muscle tissue.
41. Takken T, Terlingen HC, Helders PJM, Pruijs H, van Der Ent CK, Engelbert RHH. Cardiopulmonary fitness and muscle strength in patients with osteogenesis imperfecta type I. J Pediatr. 2004;145: 813–8.
42. Caudill A, Flanagan A, Hassani S, Graf A, Bajorunaite R, Harris G, et al. Ankle strength and functional limitations in children and adolescents with type I osteogenesis imperfecta. Pediatr Phys Ther. 2010;22: 288–95.
43. Pokidysheva E, Mizuno K, Bächinger HP. The collagen folding machinery: biosynthesis and post-translational modifications of collagens. Osteogenes Imperfecta 2014;57–70.
44. Gentry BA, Ferreira JA, McCambridge AJ, Brown M, Phillips CL. Skeletal muscle weakness in osteogenesis imperfecta mice. Matrix Biol. 2010;29:638–44.
45. Guven A, Al-Rijjal RA, BinEssa HA, Dogan D, Kor Y, Zou M, et al. Mutational analysis of PHEX , FGF23 and CLCN5 in patients with hypophosphataemic rickets. Clin Endocrinol. 2017;87: 103–12.
46. Veilleux L-N, Cheung M, Ben Amor M, Rauch F. Abnormalities in muscle density and muscle function in Hypophosphatemic rickets. J Clin Endocrinol Metab. 2012;97:E1492–8.

47. Veilleux L-N, Cheung MS, Glorieux FH, Rauch F. The musclebone relationship in X-linked Hypophosphatemic rickets. J Clin Endocrinol Metab. 2013;98:E990–5.
48. Wacker MJ, Touchberry CD, Silswal N, Brotto L, Elmore CJ, Bonewald LF, et al. Skeletal muscle, but not cardiovascular function, Is Altered in a Mouse Model of Autosomal Recessive Hypophosphatemic Rickets. Front Physiol. 2016;7:173.
49. Ma J, McMillan HJ, Karagüzel G, Goodin C, Wasson J, Matzinger MA, et al. The time to and determinants of first fractures in boys with Duchenne muscular dystrophy. Osteoporos Int. 2017;28: 597–608.
50. Trinh A, Wong P, Brown J, Hennel S, Ebeling PR, Fuller PJ, et al. Fractures in spina bifida from childhood to young adulthood. Osteoporos Int. 2017;28:399–406.
51. Mughal MZ. Fractures in children with cerebral palsy. Curr Osteoporos Rep. 2014;12:313–8.
52. Hamrick MW. The skeletal muscle secretome: an emerging player in muscle–bone crosstalk. Bonekey Rep. 2012;1:60.
53. Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, et al. A mutation in the Myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. PLoS Genet. 2007;3:e79.
54. Williams NG, Interlichia JP, Jackson MF, Hwang D, Cohen P, Rodgers BD. Endocrine actions of Myostatin: systemic regulation of the IGF and IGF binding protein Axis. Endocrinology. 2011;
55. Elkasrawy MN, Hamrick MW. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J Musculoskelet Neuronal Interact. 2010;10:56–63.
56. Mendias CL, Bakhurin KI, Gumucio JP, Shallal-Ayzin MV, Davis CS, Faulkner JA. Haploinsufficiency of myostatin protects against aging-related declines in muscle function and enhances the longevity of mice. Aging Cell. 2015;14:704–6. https://doi.org/10. 1111/acel.12339.
57. Bialek P, Parkington J, Li X, Gavin D, Wallace C, Zhang J, et al. A myostatin and activin decoy receptor enhances bone formation in mice. Bone. 2014;60:162–71. https://doi.org/10.1016/j.bone. 2013.12.002.
58. Kamiya N, Kaartinen VM, Mishina Y. Loss-of-function of ACVR1 in osteoblasts increases bone mass and activates canonical Wnt signaling through suppression of Wnt inhibitors SOST and DKK1. Biochem Biophys Res Commun. 2011;414: 326–30.
59. •• Qin Y, Peng Y, Zhao W, Pan J, Ksiezak-Reding H, Cardozo C, et al. Myostatin inhibits osteoblastic differentiation by suppressing osteocyte-derived exosomal microRNA-218: A novel mechanism in muscle-bone communication. J Biol Chem. 2017;292:11021– 33. This study reported that myostatin promotes expression of several bone regulators such as SOST, DKK1, and RANKL in cultured osteocytic cells, which in turn exerts an inhibitory effect on osteoblast differentiation.
60. Arnold MA, Kim Y, Czubryt MP, Phan D, McAnally J, Qi X, et al. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell. 2007;12:377–89.
61. Consalvi S, Mozzetta C, Bettica P, Germani M, Fiorentini F, Del Bene F, et al. Preclinical studies in the mdx mouse model of duchenne muscular dystrophy with the histone deacetylase inhibitor givinostat. Mol Med. 2013;19:79–87.
62. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol. 2012;8:457– 65.

63. Tagliaferri C, Wittrant Y, Davicco M-J, Walrand S, Coxam V. Muscle and bone, two interconnected tissues. Ageing Res Rev. 2015;21:55–70.
64. Chung S, Dzeja PP, Faustino RS, Perez-Terzic C, Behfar A, Terzic A. Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. Nat Clin Pract Cardiovasc Med. 2007;4:S60–7.
65. Rochard P, Rodier A, Casas F, Cassar-Malek I, Marchal-Victorion S, Daury L, et al. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. J Biol Chem. 2000;275:2733–44.
66. Ehlen JC, Brager AJ, Baggs J, Pinckney L, Gray CL, DeBruyne JP, et al. Bmal1 function in skeletal muscle regulates sleep. Elife 2017;6.
67. Samsa WE, Vasanji A, Midura RJ, Kondratov RV. Deficiency of circadian clock protein BMAL1 in mice results in a low bone mass phenotype. Bone. 2016;84:194–203.
68. Nelson MR, Tipney H, Painter JL, Shen J, Nicoletti P, Shen Y, et al. The support of human genetic evidence for approved drug indications. Nat Genet. 2015;47:856–60.
69. Wöhrle S, Henninger C, Bonny O, Thuery A, Beluch N, Hynes NE, et al. Pharmacological inhibition of fibroblast growth factor (FGF) receptor signaling ameliorates FGF23-mediated hypophosphatemic rickets. J Bone Miner Res. 2013;28:899–911.
70. Carpenter TO, Imel EA, Ruppe MD, Weber TJ, Klausner MA, Wooddell MM, et al. Randomized trial of the anti-FGF23 antibody KRN23 in X-linked hypophosphatemia. J Clin Invest. 2014;124: 1587–97.
71. Imel EA, Zhang X, Ruppe MD, Weber TJ, Klausner MA, Ito T, et al. Prolonged correction of serum phosphorus in adults with Xlinked hypophosphatemia using monthly doses of KRN23. J Clin Endocrinol Metab. 2015;100:2565–73.
72. Potthoff MJ, Wu H, Arnold MA, Shelton JM, Backs J, McAnally J, et al. Histone deacetylase degradation andMEF2 activation promote the formation of slow-twitch myofibers. J Clin Invest. 2007;117:2459–67.
73. Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, et al. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature. 2005;434:514–20.
74. Oury F, Sumara G, Sumara O, Ferron M, Chang H, Smith CE, et al. Endocrine regulation of male fertility by the skeleton. Cell. 2011;144:796–809.





4.2

Investigation of shared genetics between bone mineral density and lean mass in elderly

Katerina Trajanoska, Niki L Dimou, David Karasik, Fernando Rivadeneira on behalf of GEFOS consortium

In preparation

ABSTRACT

Background: The intimate relationship between bone and muscle is governed by various mechanical and biochemical interactions; regulated by a complex network of pleiotropic genes within common biological pathways. We aimed to assess pleiotropy between bone and muscle by performing a bivariate genome-wide association study (GWAS).

Methods: We included 15 cohorts from populations across Europe, the US and Australia (N_{total}= 30,531). Total body BMD (TB-BMD; g/cm^2) and lean mass (TB-LM; kg) were measured in all cohorts using DXA. Genetic variants were imputed to the Haplotype Reference Consortium (HRC) reference panel. Each cohort performed univariate GWAS adjusting for age, gender, height, fat percentage and principal components using 8,027,751 SNPs. After GWAS meta-analysis genome-wide significance (GWS) was set at $P<5\times10^{-8}$.

Results: The univariate TB-BMD GWAS identified 18 signals all mapping to known BMD loci. Only four loci were associated with TB-LM, three of which were novel associations mapping to ZC3H11B (rs1810144-G, beta=0.06SD) *SPEG* (rs1810144-C, beta=0.05SD) and *MYPN* (rs12415105-G, beta=0.04SD). Both *SPEG* and *MYPN* have evidence from animal models for implication in muscle biology. Notably, the bivariate meta-analysis yielded 17 pleiotropic signals from 15 different loci mapping in or near *MEF2C,* XYLB/ACVR2B, *PPP6R3/LRP5, LRP4* and *SLC27A6* among others, linked with both bone and muscle biology.

Conclusion: Our findings provide basis for future functional assessments aimed at unravelling biological mechanisms underlying complex bone-muscle interactions; with the potential of pin-pointing strategies for the joint prevention and intervention of osteoporosis and sarcopenia.

INTRODUCTION

The intimate relationship between bone and muscle is governed by various mechanical and biochemical interactions. To begin with, bone and muscle precursors originate from the mesenchymal stem cells, go through well-orchestrated, multifaceted mechanisms of cell proliferation and differentiation, and eventually give rise to bone or muscle tissue. The bone tissue begins to experience mechanical stimulations during embryogenesis with the start of the first muscle contractions; contributing to fetal bone development and growth. The mechanical stimulation and loading continues after birth and is pivotal for reaching peak bone mass in early adulthood and maintaining bone mass later in life.[1] At the same time, bone and muscle tissues release variety of endocrine, paracrine, and autocrine factors which influence the surrounding tissues or each other.[2] All these mechanisms are regulated by complex network of genes that can affect either one or both tissues. Therefore, bone and muscle may share common biological pathways i.e. pleiotropy; a genetic variant that influences multiple traits.

Multitude genetic factors have been associated with the variation of bone and lean mass in the general population. Several of these loci such as *MC4R, FTO a*nd *MGMT* harbor genetic variants associated with both bone and muscle;[3,4,5] indicating possible pleiotropy. However, simple cross-phenotype association is not an evidence of pleiotropy. Although pleiotropy is a challenging to be determine several approaches have been developed which can be generally classified as multivariate or univariate. [6]The former approach requires all individuals included in the study to have phenotype information of all traits of interest. On the other hand, the univariate approach combines summary statistics of single-trait GWAS. Combining summary statistics several genes have been proposed to have pleotropic effect on both bone and muscle traits such as *METTL21C* and *GLYAT.*[7,8] However, these results are yet to be replicated in large-scale efforts and validated by functional studies. Recently, a large pediatric effort presented a novel gene, *SREBF1,* exerting opposite effects on total body bone and muscle mass.[9] We have now extended this effort by including individuals across different age categories in order to identify novel genes affecting bone and muscle using much more powerful study settings.

SUBJECTS AND METHODS

Study Populations

This effort included 15 cohorts from populations across Europe, the USA and Australia from the GEFOS consortium. The majority of the cohorts were of European ancestry with only two cohorts with admixed background (GENR and RAINE). In addition, two

out of the 15 cohorts were pediatric cohorts (GENR and ALSPAC). Each of the studies was approved by their local Medical Ethical Committee and only participants with written informed consent were included.

Phenotype Assessment

Total body BMD (g/cm^2) and total body lean mass (kg) were assessed in all cohorts using dual-energy X-ray absorptiometry (DXA). The pediatric cohorts used total body less head as recommended by the International society for clinical densitometry.

Genotyping and Imputation

All cohorts used commercially available genome-wide arrays to genotyped their participants. All cohorts did pre-imputation quality control using already established protocols. SNPs were imputed to the Haplotype Reference Consortium (HRC) reference panel[10] (build 37). The imputation was done using free imputation servers (Michigan or Sanger) and resulted in approximately 40,000,000 SNPs.

Univariate Genome-wide Association Analysis

Each study preformed linear regression models for both BMD and lean mass adjusted for age, gender, height, fat percentage and principal components. For both traits residuals were computed separately by sex while in the family studies sex was included as a covariate in the model. Prior meta-analysis quality controls (QC) was performed with easy QC.[11] Genetic variants with low imputation quality (r^2<0.3) or low minor allele frequency (MAF<0.01) were excluded. Each cohort was tested for possible spurious inflation by computing lambda and visual inspection of the QQ plots. Finally, all cohorts were combined using inverse variance weighted fixed meta-analysis in Metal.[12] Genome wide significant threshold was set to $5x10^{-8}$. Independent SNPs were defined using clumping methods in Plink (parameters of --clump-p1 5e-8 --clump-kb 500 --clump-r2 0.2).

Multi-trait Analysis

Next, we preformed multi-trait analysis of genome-wide association summary statistics using MTAG in order to boost statistical power to detect additional genetic loci from the univariate GWAS for each trait analyzed.[13] The advantages of this method are that 1) statistics don't need to come from independent efforts and overlap does not influence the results as it is based on the LD score repression which accounts for sample overlap, 2) it can included more than two traits and 3) it is computationally very quick. Finally, if a SNP is null for one trait but not for the other can lead to false positives however MTAG also account for this credibility.

Bivariate Analysis

Finally we used the sum rank method, a novel approach, to test for possible pleiotropic variants influencing both BMD and lean mass.[14] The sum ranking method is a simple statistical test that is based on the GWAS summary p-values which covert to an individual rank of every variant for a given trait and computes a final pleiotropy p-values and is robust to extreme values.

Characterization and Annotation of the Genomic Loci

First, for each of the lead SNP we evaluated the DNA features and regulatory elements in noncoding regions of the human genome using RegulomeDB.[15] Next we used the CAAD tool to score the deleteriousness of the lead SNPs.[16,17] The higher the score the more deleterious the variant is. We then searched the closest genes in the Mouse Genome Informatics (MGI) database to explore presence of musculoskeletal phenotypes in mice mutants (http://www.informatics.jax.org).[18] Finally, we performed eQTL analysis using the online GTEx database based on 54 non-diseased tissue sites across nearly 1000 individuals (https://gtexportal.org/home/).[19]

RESULTS

Univariate and Multi-trait Analyses

In the univariate GWAS meta-analysis of TB-BMD 2,081 SNPs surpassed the genome-wide significant threshold (**Figure 1, upper half; Supplementary Table 1**) which mapped to 18 different genomic loci. All discovered loci are known BMD loci and the top genetic variants mapped in or near *WNT4* (rs10493013-C, beta=0.09SD), *WLS* (rs2566752-C, beta=0.05SD), *EN1* (rs141795717-A, beta=0.21 SD), *CSRNP3/GALNT3* (rs6719426-G, beta=0.05SD), *DGKD* (rs7584554-G,beta=0.05SD), *CTNNB1* (rs7609599-G, beta=0.05SD), *RSPO3* (rs4580892-T, beta=0.05SD), *C7orf76/SHFM1* (rs7787512-T, beta=0.06SD), *CPED1/WNT16* (rs2908004-A, beta=0.10SD), *TNFRSF11B* (rs7014574-C, beta=0.05SD), *MBL2* (rs7902708-G, beta=0.07SD), *CCDC34* (rs60212556-C, beta=0.08SD), *C11orf49* (rs79232684-A, beta=0.09SD), *PPP6R3* (rs4752957-A, beta=0.07SD), *TMEM135* (rs618926-C, beta=0.05SD), *SP7* (rs10747668-C, beta=0.06SD), *FABP3P2* (rs9533094-A, beta=0.05SD) and *MEOX1/DUSP3* (rs66838809-A, beta=0.09SD). On the other hand, only four loci harbored GWS variants for TB-LM which mapped near or to *ZC3H11B* (rs1810144-G, beta=0.06SD), *SPEG* (rs1810144-C, beta=0.05SD) MYPN (rs12415105-G, beta=0.04SD) and *FTO* (rs56094641-G, beta=0.05SD) (**Figure 2, upper half; Supplementary Table 1**). Both meta-analyses did not show any inflation in the test statistics (**Supplementary Figure 1A and 1B**). In the multi-trait analysis, the genetic correlation between TB-BMD and

TB-LM was 0.40. Although moderate, no additional SNPs reached GWS for TB-BMD (**Figure 1, lower half**) while for TB-LM only one additional variant surpassed the GWAS threshold (**Figure 2, lower half**) which mapped to *PPP6R3*; known TB-BMD loci that have not been reported with TB-LM before.

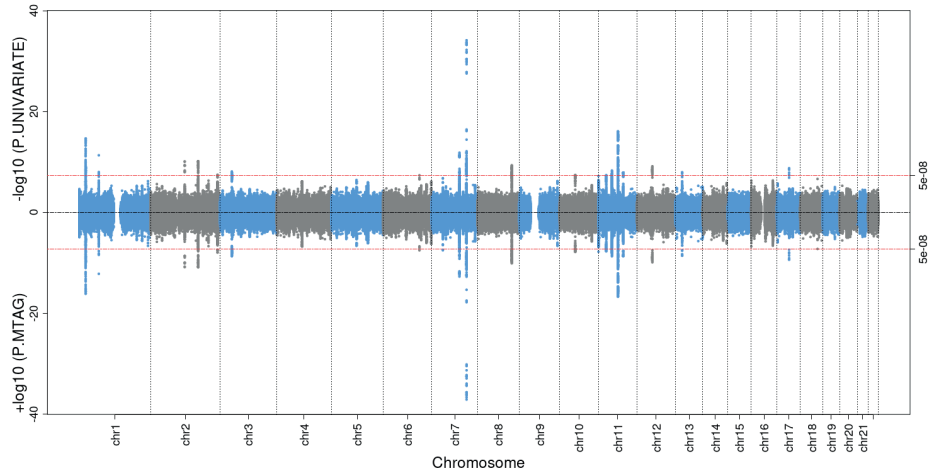


Figure 1 | Miami plot displaying the univariate –log10 (P values) (top half) and multi-trait GWAS (MTAG) –log10 (P values) (bottom half) for the SNP associations with TB-BMD. Dashed red lines mark the GWS threshold ($P < 5 \times 10^{-08}$).

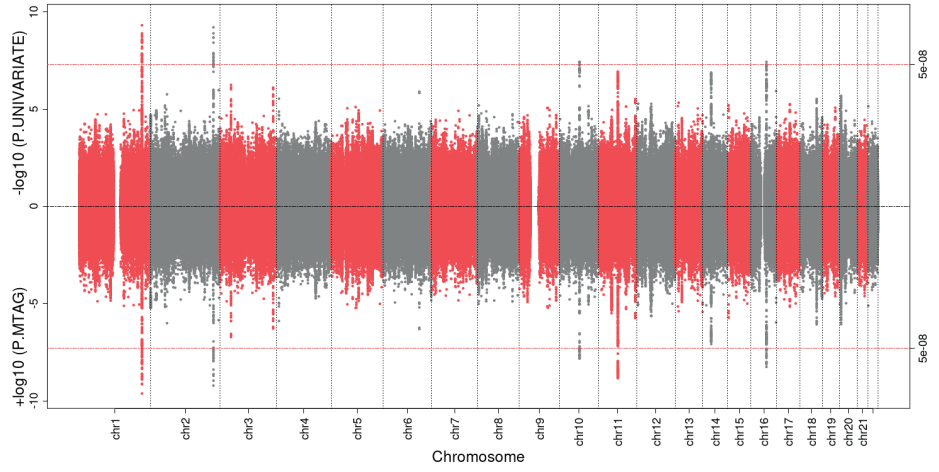


Figure 2 | Miami plot displaying the univariate –log10 (P values) (top half) and multi-trait GWAS (MTAG) –log10 (P values) (bottom half) for the SNP associations with TB-LM. Dashed red lines mark the GWS threshold ($P < 5 \times 10^{-08}$).

Table 1 | Lead SNPs for the 17 bivariate association signals with TB-BMD and TB-LM.

						Total Body BMD			Total Body LM			Bivariate	Closest
Locus	**SNP**	**BP**	**EA**	**NEA**	**EAF**	**Effect**	**se**	**P**	**Effect**	**SE**	**P**	**P**	**Gene**
1p13.3	rs4970731	109995459	T	C	0.30	0.04	0.01	$4.74x10^{-5}$	0.04	0.01	$6.23x10^{-5}$	$6.01x10^{-9}$	*SYPL2/PSMA5*
1q41	rs10779360	219731273	T	C	0.61	0.03	0.01	$2.56x10^{-4}$	0.04	0.01	$2.18x10^{-7}$	$3.27x10^{-8}$	*ZC3H11B*
3p22.2	rs1381798	38437471	A	G	0.29	0.03	0.01	$1.21x10^{-4}$	0.04	0.01	$1.32x10^{-5}$	$8.96x10^{-9}$	*XYLB*
5q14.3	rs254777	88008871	C	G	0.74	0.05	0.01	$9.15x10^{-7}$	0.04	0.01	$1.59x10^{-5}$	$1.41x10^{-10}$	*MEF2C*
5q23.3	rs7706547	128000893	T	C	0.16	-0.05	0.01	$2.97x10^{-6}$	-0.04	0.01	$1.71x10^{-4}$	$1.52x10^{-8}$	*SLC27A6*
6q22.33	rs9491689	127398595	A	C	0.28	0.05	0.01	$1.41x10^{-7}$	0.04	0.01	$5.55x10^{-5}$	$1.55x10^{-9}$	*RSPO3*
6q27	rs2609312	168816483	A	C	0.59	-0.04	0.01	$4.10x10^{-6}$	-0.03	0.01	$8.39x10^{-5}$	$3.87x10^{-9}$	*SMOC2*
11q13.2	rs7113287	68372186	A	T	0.73	0.07	0.01	$1.17x10^{-15}$	0.05	0.01	$1.10x10^{-7}$	$6.00x10^{-15}$	*PPP6R3*
11p11.2	rs61882720	46538988	A	C	0.17	0.06	0.01	$3.92x10^{-7}$	0.05	0.01	$2.48x10^{-5}$	$3.17x10^{-10}$	*AMBRA1*
11p11.2	rs12806687	46924665	C	G	0.35	0.05	0.01	$1.26x10^{-7}$	0.03	0.01	$7.96x10^{-5}$	$3.17x10^{-9}$	*LRP4*
11p11.2	rs1976143	47225534	A	G	0.67	-0.04	0.01	$3.09x10^{-7}$	-0.03	0.01	$1.23x10^{-4}$	$7.64x10^{-9}$	*PACSIN3/DDB2*
12q24.31	rs10846578	124400278	T	C	0.28	-0.04	0.01	$6.55x10^{-5}$	-0.04	0.01	$4.83x10^{-5}$	$6.47x10^{-9}$	*DNAH10*
12q13.11	rs11168357	48412138	A	G	0.23	-0.04	0.01	$1.61x10^{-4}$	-0.04	0.01	$5.50x10^{-5}$	$2.34x10^{-8}$	*COL2A1*
13q34	rs2701321	115058005	A	G	0.09	0.06	0.01	$1.05x10^{-5}$	0.06	0.01	$2.05x10^{-5}$	$4.79x10^{-10}$	*UPF3A*
13q14.11	rs9590675	42749192	T	G	0.40	-0.03	0.01	$2.15x10^{-4}$	-0.03	0.01	$4.40x10^{-5}$	$3.35x10^{-8}$	*DGKH*
16q12.2	rs55872725	53809123	T	C	0.39	0.03	0.01	$2.96x10^{-5}$	0.05	0.01	$5.76x10^{-8}$	$4.41x10^{-10}$	*FTO*
16p13.13	rs56092102	10671035	T	G	0.09	-0.07	0.01	$7.56x10^{-6}$	-0.05	0.01	$2.39x10^{-4}$	$3.03x10^{-8}$	*EMP2*

EA=effect allele; NEA= non-effect allele; Beta coefficients and allele frequencies (EAF) are reported for the EA.

Table 2 | Functional annotation of the leads SNPs

SNP	Closest Gene	GTEx tissue	GTEx Gene	RegulomeDB	CAAD	Mouse Models Tissue	Other phenotypes*
rs4970731	*SYPL2/PSMA5*	Muscle-Skeletal, Testis, Lungs	*SYPL2, MYBPHL*	-	3.30	Muscle, Growth	glomerular filtration rate, BMI, intelligence
rs10779360	*ZC3H11B*	-	-	-	0.17	Aging	BMI., adiposity, smoking behavior
rs1381798	*XYLB*	Thyroid, Cerebellum, Pancreas	*EXOG*	5	8.73	Skeleton, Growth	glomerular filtration rate
rs254777	*MEF2C*	-	-	-	1.82	Skeleton, Muscle, Growth, Aging	platelet count, erythrocyte count, height
rs7706547	*SLC27A6*	Adrenal Gland, Hear, Skin	*FBN2*	6	1.30	-	red blood cell distribution width, atrial fibrillation
rs9491689	*RSPO3*	-	-	-	1.92	Muscle, Aging	BMI, smoking behavior
rs2609312	*SMOC2*	Muscle-Skeletal, Thyroid	*SMOC2*	5	2.25	Skeleton	Height, pulse pressure, Vitiligo
rs7113287	*PPP6R3*	Whole Blood, Tibial Nerve	*GAL*	-	2.04	-	educational attainment, red cell distribution width
rs61882720	*AMBRA1*	Skin. Esophagus -Muscularis	*ATG13*	-	1.03	Aging	schizophrenia
rs12806687	*LRP4*	Muscle-Skeletal, Pancreas, Tibial Nerve	*LRP4, ARHGAP1*	4	4.40	Skeleton, Growth	Thrombosis, systolic blood pressure, risk-taking behavior
rs1976143	*PACSIN3/DDB2*	Tibial Nerve, Pancreas, Skin	*LRP4, DDB2*	5	0.11	Growth, Aging	Educational attainment, HDL cholesterol
rs10846578	*DNAH10*	-	-	5	2.55	Aging	BMI, grip strength, height
rs11168357	*COL2A1*	Thyroid. Skin, Lungs	*RP1-228P16.1*	4	0.38	Skeleton, Growth	Height
rs2701321	*UPF3A*	Muscle-Skeletal	*CDC16*	-	1.15	-	CVD, Pulse pressure, Hodgkin lymphoma
rs9590675	*DGKH*	-	-	4	14.84	-	glomerular filtration rate
rs55872725	*FTO*	Muscle-Skeletal	*FTO*	4	1.57	Skeleton	BMI
rs56092102	*EMP2*	-	-	5	0.30	-	facial asymmetry measurement, FEV/FEC ratio, erythrocyte count

*this column indicates if the gene has been related with other phenotypes.

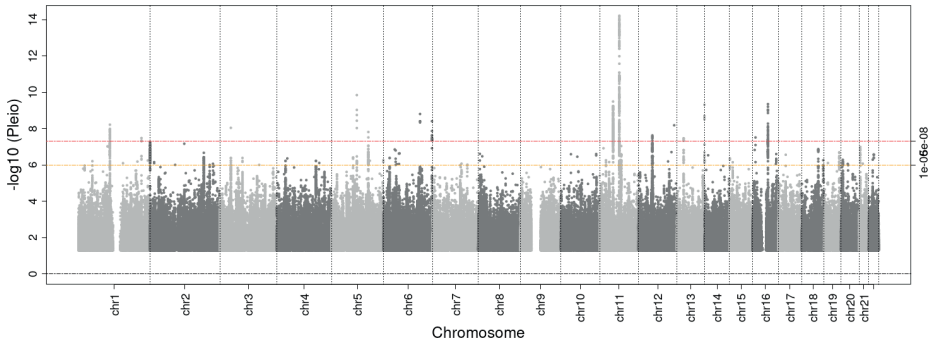


Figure 3 | Manhattan plot displaying the bivariate –log10 (P values) for the SNP associations with TB-BMD and TB-LM. Dashed red and yellow lines mark the GWS threshold ($P < 5\times10^{-08}$) and suggestive threshold ($P < 1\times10^{-6}$), respectively.

Bivariate Analysis

The bivariate meta-analysis yielded 17 GWAS hits mapping to 15 different loci (**Table 1; Supplementary Figure 2a-p**) Eleven variants were intronic and mapped to *ZC3H11B* (rs10779360-T), *XYLB* (rs1381798-A), *SLC27A6* (rs7706547-C), *PPP6R3* (rs7113287-A), *AMBRA1* (rs61882720-A), *LRP4* (rs12806687-C), *DNAH10* (rs10846578-C), *UPF3A* (rs2701321-A), *DGKN* (rs9590675-G), FTO (rs55872725-T) and *EMP2* (rs56092102-G). The other six were intergenic mapping near *SYPL2/PSMA5* (rs4970731-T, kb=13.64), *MEF2C* (rs254777-C, kb=5.10), *RSPO3* (rs9491689-A, kb=41.15), *SMOC2* (rs2609312-C, kb=25.35), PACSIN3 (rs1976143-G, kb=17.52) and *COL2A1* (rs11168357-G, kb=13.85). The top most significant locus (*PPP6R3*) displayed stronger association with TB-BMD and was only suggestive for TB-LM in the univariate GWAS analyses. All variants exhibited the same direction of effect on both lean mass and BMD.

Characterization and Annotation of the Genomic Loci

All of the lead variants had high RegulomeDB score (>=4) indicating that the SNPs are less likely to be functional, thus, do not affect gene biding and expression. Similarly, all lead SNPs had low CAAD score; demonstrating they are not deleterious. In the MGI database only the Mef2c knock-out (KO) mice presented with both muscle and bone abnormalities such as dilated cardiomyopathy, abnormal skeletal muscle fiber type ratio, decreased bone length, abnormal trabecular bone morphology abnormal endochondral bone ossification among others. In addition, only the Sypl2 and Rspo3 KO mice presented only with muscle abnormalities, whereas, Xylb, Smoc2, Lrp4, Col2a1, and Fto KO mice had only skeletal deformities. Finally, five of the lead SNPs (rs4970731, rs2609312, rs12806687, rs2701321, rs55872725) were cis-eQTL variants that can regulate gene expression (*SYPL2, SMOC2, LRP4, CDC16,* and *FTO*

respectively) in skeletal muscle tissue. However, the variants also showed significant eQTLs in other tissues such as lungs, testis, thyroid and skin.

DISCUSSION

We performed a large scale bivariate GWAS between TB-BMD and TB-LM including up to 30,000 individuals and discovered 15 loci harboring variants associated with both TB-BMD and TB-LM. The top variant at the 5q14.3 locus mapped near the Myocyte enhancer factor–2C (*MEF2C*) gene that have been previously linked to various aspects of bone and muscle biology. On top of this we also identified other genes with potential pleiotropic effects such as *XYLB, PPP6R3, COL2A1* and *FTO*. Finally, our univariate TB-LM GWAS yielded three novel lean mass associated variants mapping to 1q41 (*ZCH311B*), 2q35 (*SPEG*) and 10q21.3 (*MYPN*).

The 5q14.3 locus harbored the strongest pleiotropic signal (rs254777-C) which maps near ***MEF2C*** (5.10kb). This gene belongs to the MEF2 transcription factor family which encodes four muscle-specific transcription factors: Mef2a, -b, -c, and -d, that regulate different aspects of skeletal, cardiac and smooth muscle cell growth and differentiation.[20,21] In mice, Mef2c is the first expressed gene during embryogenesis from the mef2[22] and it is crucial for myocardiogenesis and morphogenesis[23]. The mef2 factors are expressed in all developing muscle cell types[22] and are important for muscle fiber differentiation, elongation and fusion;[24] thus, may influence muscle mass variation in the general population. The loss of Mef2c and Mef2d proteins causes a defect in myofibrillogenesis and sarcomere formation.[25] Notably, in matured muscles the mef2 factors have been associated with improved skeletal muscle regeneration after muscle injury;[26] fortifying the important role of the MEF2 family in muscle biology. *MEF2C* is also a known BMD locus[27,28] which was suggestive GWAS signal in our univariate TB-BMD GWAS. In mice, it has been shown to promote chondrocyte hypertrophy and bone development.[29] Furthermore, *MEF2C* also controls the cortical expression of Sfrp2 and Sfrp3 (WNT inhibitors) and it is vital for adequate Sost expression;[30] important regulator of bone formation. Nevertheless, the increased bone mass in Mef2c mutants is not directly related to the reduced Sost expression.[30] Overall, mutations in the *MEFC2* gene lead to both impaired muscle and bone structure. Elucidating the underlying pathways may be crucial for preventing and treating osteoporosis and sarcopenia simultaneously.

Another interesting pleiotropic region is the 11q13.2 locus harboring GWAS variants mapping to the ***PPP6R3*** gene which are also in high LD (r2>0.8) with variants from the *LRP5* gene region. The *PPP6R3/LRP5* region is a known BMD locus and have been associated with both bone and lean mass in children.[28] *LRP5* is an important WNT

co-receptor and it is involved in bone formation and mechanotransduction.[31] Mutations in the LRP5 gene case *autosomal recessive osteoporosis-pseudoglioma syndrome* characterized by infancy-onset vision loss and severe juvenile osteoporosis[32] but also muscular hypotonia and ligamentous laxity.[33] However, the exact effects of LPR5 on muscle mass remain unknown. Furthermore, on chromosome 11 there is additional locus (11p11.2) that harbor four possible pleiotropic genes such as ***LPR4, AMBRA1, PACSIN3*** and ***DDB2***. The ***LRP4*** gene is an important mediator of the SOST-dependent inhibition of bone formation.[34] In addition, *LRP4* is essential for neuromuscular junction (NMJ) formation and maintenance.[35] Mutations in this gene have been associated with i) *Cerani-Lenz syndrome* characterized by syndactyly and oligodactyly of the fingers and toes with malformations of the forearm bones and lower limbs[36] and ii) *Myasthenic syndrome* characterized by failure in neuromuscular transmission resulting in increased fatigue and generalized muscle weakness.[37] Next, ***AMBRA1*** is a known BMD gene and the top SNP is in LD (r^2>0.8) with variants mapping to the surrounding genes also related to BMD and variety of neurological traits. The third lead SNP in this locus was intergenic mapping between the ***PACSIN3*** and ***DDB2*.** Both genes have been previously related to total body and heel BMD.[28,38] The exact mechanisms of action of these genes on bone and muscle are yet to be determine.

The 1p13.3 GWAS signals are intergenic and map between the ***SYPL2*** and ***PSMA5*** gene regions that have been previously associated with glomerular filtrationrate,[39] human intelligence[40] and BMI[41]. The lead SNP from this locus is in strong LD (r^2>0.8) with variants mapping to the *SORT1* gene that has been recently linked with variation in appendicular lean mass[42] and variants mapping to the *MYBPHL* gene which have been associated with increased risk for cardiomyopathy and arrhythmia in humans.[43] This gene is mainly expressed in heart ventricles,[43,44] whereas, its paralog *MYBPH* is highly expressed in skeletal muscles only[43,44]. Several GWAS variants also mapped to the ***XYLB*** gene. The lead variant from this locus is in moderate LD with variants mapping to the *ACVr2B* gene. This gene encodes the activin type 2 receptor protein and it is part of the TGF-beta superfamily of signaling proteins. Administration of soluble form of ACVR2B in wild-type mice leads to substantial increase in muscle mass by up to 60%[45] as well as increase in bone and muscle mass in a mouse model of osteogenesis imperfect[46] and in dystrophic mice.[47] In addition, mice lacking ACVR2B and ACVR2A demonstrated sustained increases in trabecular bone volume.[48] Next, the top signal in 5q23.3 is located in the vicinity of the ***SLC27A6*** gene which encodes several members of the fatty acid transport protein family. Importantly, this gene is part of the PPAR signally pathway. Par is an important regulator of bone turnover in both mice and humans.[49] On the other hand, PPARα is expressed in skeletal muscles in both humans and mice and its activation in rare cases (<1%) may lead to muscle weakness, muscle pain and even breakdown of muscle.[50] Furthermore, ***RSPO3*** may

also have pleiotropic effects on both bone and muscle. This gene is a well-known BMD and fracture risk; however, it has not been associated with muscle mass before. What we know so far is that the Rspo3 protein promotes angioblast, vascular and cardiac development.[51,52] Similar, the top variants from the 6q27 locus mapped to the ***SMOC2*** gene which have been also implicated in angiogenesis. Mutations is this gene have been related to Dentin Dysplasia.[53] ***DNAH10*** have been previously associated with appendicular lean mass[42] and BMI.[54] There is no evidence for association with BMD or other bone phenotypes. On the other hand, the ***COL2A1*** gene located on 12q13.11 is a well-established bone region. In addition, the lead SNP from this region is in a moderate LD (r^2>0.6) with SNPs associated with appendicular lean mass.[42] *COL1A1* is relevant for the production of type II collagen which is found primary in the bone cartilage but also in the muscle tissue among others. Mutations in this gene are characterized by variety of skeletal phenotypes such as achondrogenesis type II and hypochondrogenesis.[55] Two additional pleiotropic signals mapped on two distinct loci on chromosome 13. The signals on 13q14.11 maps to the ***DGKH*** gene which has been associated with glomerular filtration rate[56] and height.[57] Now we also show evidence for its possible implication in bone and muscle mass variation. Notably, its paralog *DGKD* have been associated with bone density and calcium levels among other traits.[28,58] The second locus 13q34 contained the ***UPF3A*** gene which is involved in both mRNA nuclear export and mRNA surveillance. Its effects on bone and muscle tissue are still unknown. Last but not least chromosome 16 also harbored two loci with possible pleiotropic effects. On 16q12.2 the GWS variants mapped to ***FTO*** a well-known obesity gene which have been also related with both BMD and muscle mass in previous GWAS efforts;[3,38] indicating that *FTO* may be important link between bone, muscle and fat tissue. Finally, the signals on the second locus (16p13.13) mapped near ***EMP2*** a known heel BMD gene[38] that have not been related to any muscle trait till date. Notably, this gene is a positive relator of the vascular endothelial growth factor A (VEGF). Interestingly, we discovered several genes related to vascular development and angiogenesis. Given bone and muscle tissue are highly vascularized, these genes may play important role in osteo- and or myogenesis.

Our univariate analysis for TB-BMD yielded 18 loci that have been previously reported by large GWAS efforts.[27,28,38] Notably, we identified three novel loci associated with TB-LM which have not been previously reported. The most significant variant on locus 2q35 mapped to ***SPEG*** (Striated muscle preferentially expressed protein kinase) which encodes a protein similar to the members of the myosin light chain kinase family and it is required for myocyte cytoskeletal development.[59] Mutations in this gene have been associated with centronuclear myopathy which is characterized by muscle weakness and wasting[60] and often occur due to interaction between *SPEG* and myotubularin (*MTM1*). In addition, Speg-KO mice were weaker and smaller compared

to their wild-mice controls.[61] Next, we also identified the *MYPN* (Myopalladin) gene as novel muscle-related gene which have been previously associated with cardiomyopathy and nemaline myopathy.[62] *MYPN* is relevant for the interaction between the sarcomere and nucleus in cardiac and skeletal muscles.[63,64] In the MGI database Mypn mice-KOs are characterized by abnormal myocardial fiber morphology, abnormal cardiac muscle relaxation, abnormal intercalated disk morphology, abnormal Z line morphology, and myopathy.

Our study is the largest bivariate GWAS meta-analysis that have been done so far on TB-BMD and TB-LM providing adequate power to detect loci with pleiotropic effects. In addition, we used a novel rank-based approach to detect these pleiotropic variants and our findings should not be biased by strong association with one of the phenotypes; producing false positive results. Finally, both TB-BMD and TB-LM were measured using the same technique (DXA) and each study used the same scanner. We cannot rule out possible false positive findings. Replication and functional studies need to confirm and further characterize our findings. Currently we are working on embarking meta-analysis using individual level data in order to prove more robust evidence of pleiotropy.

In summary, through bivariate genome-wide association study we were able to provide strong evidence for genetic overlap between TB-BMD and TB-LM in the general population. Our findings provide basis for future functional studies aimed to unravel the biological mechanisms behind the complex bone-muscle interaction; bring us one step closer to the joint prevention and intervention of osteoporosis and sarcopenia.

REFERENCES

1. Bergmann, P. *et al.* Loading and Skeletal Development and Maintenance. *J. Osteoporos.* **2011**, 1–15 (2011).
2. Karsenty, G. & Olson, E. N. Leading Edge Review Bone and Muscle Endocrine Functions: Unexpected Paradigms of Inter-organ Communication. (2016). doi:10.1016/j.cell.2016.02.043
3. Zillikens, M. C. *et al.* Large meta-analysis of genome-wide association studies identifies five loci for lean body mass. *Nat. Commun.* **8**, 80 (2017).
4. Willems, S. M. *et al.* Large-scale GWAS identifies multiple loci for hand grip strength providing biological insights into muscular fitness. **8**, 16015 (2017).
5. Kemp, J. P. *et al.* Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis. *Nat. Genet.* **49**, 1468–1475 (2017).
6. Hackinger, S. & Zeggini, E. Statistical methods to detect pleiotropy in human complex traits. *Open Biol.* **7**, 170125 (2017).
7. Huang, J. *et al. METTL21C* Is a Potential Pleiotropic Gene for Osteoporosis and Sarcopenia Acting Through the Modulation of the NF-κB Signaling Pathway. *J. Bone Miner. Res.* **29**, 1531–1540 (2014).
8. Guo, Y.-F. *et al.* Suggestion of GLYAT gene underlying variation of bone size and body lean mass as revealed by a bivariate genome-wide association study. *Hum. Genet.* **132**, 189–199 (2013).
9. Medina-Gomez, C. *et al.* Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/TOM1L2 locus. *Nat. Commun.* **8**, 121 (2017).
10. Consortium, the H. R. *et al.* A reference panel of 64,976 haplotypes for genotype imputation. *Nat. Genet.* **48**, 1279–1283 (2016).
11. Winkler, T. W. *et al.* Quality control and conduct of genome-wide association meta-analyses. *Nat. Protoc.* **9**, 1192–212 (2014).
12. Willer, C. J., Li, Y. & Abecasis, G. R. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics* **26**, 2190–2191 (2010).
13. Turley, P. *et al.* Multi-trait analysis of genome-wide association summary statistics using MTAG. *Nat. Genet.* **50**, 229–237 (2018).
14. RePub, Erasmus University Repository: Imaging genetics : Methodological approaches to overcoming high dimensional barriers. Available at: https://repub.eur.nl/pub/106452. (Accessed: 2nd January 2020)
15. Boyle, A. P. *et al.* Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res.* **22**, 1790–1797 (2012).
16. Kircher, M. *et al.* A general framework for estimating the relative pathogenicity of human genetic variants. *Nat. Genet.* **46**, 310–315 (2014).
17. Rentzsch P, Witten D, Cooper GM, Shendure J, K. M. CADD: predicting the deleteriousness of variants throughout the human genome.
18. Bult, C. J. *et al.* Mouse Genome Database (MGD) 2019. *Nucleic Acids Res.* **47**, D801–D806 (2019).
19. Carithers, L. J. *et al.* A Novel Approach to High-Quality Postmortem Tissue Procurement: The GTEx Project. *Biopreserv. Biobank.* **13**, 311–317 (2015).

20. Olson, E. N., Perry, M. & Schulz, R. A. Regulation of Muscle Differentiation by the MEF2 Family of MADS Box Transcription Factors. *Dev. Biol.* **172**, 2–14 (1995).
21. Black, B. L. & Olson, E. N. TRANSCRIPTIONAL CONTROL OF MUSCLE DEVELOPMENT BY MYOCYTE ENHANCER FACTOR-2 (MEF2) PROTEINS. *Annu. Rev. Cell Dev. Biol.* **14**, 167–196 (1998).
22. Edmondson, D. G., Lyons, G. E., Martin, J. F. & Olson, E. N. Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251–63 (1994).
23. Lin, Q., Schwarz, J., Bucana, C. & Olson, E. N. Control of Mouse Cardiac Morphogenesis and Myogenesis by Transcription Factor MEF2C. *Science (80-.).* **276**, 1404–1407 (1997).
24. Hinits, Y. & Hughes, S. M. Mef2s are required for thick filament formation in nascent muscle fibres. *Development* **134**, 2511–2519 (2007).
25. Potthoff, M. J. *et al.* Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. *Mol. Cell. Biol.* **27**, 8143–51 (2007).
26. Liu, N. *et al.* Requirement of MEF2A, C, and D for skeletal muscle regeneration. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 4109–14 (2014).
27. Estrada, K. *et al.* Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat. Genet.* **44**, 491–501 (2012).
28. Medina-Gomez, C. *et al.* Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects. *Am. J. Hum. Genet.* **102**, 88–102 (2018).
29. Arnold, M. A. *et al.* MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development. *Dev. Cell* **12**, 377–389 (2007).
30. Kramer, I., Baertschi, S., Halleux, C., Keller, H. & Kneissel, M. *Mef2c* deletion in osteocytes results in increased bone mass. *J. Bone Miner. Res.* **27**, 360–373 (2012).
31. Kang, K. S. & Robling, A. G. New Insights into Wntâ€"Lrp5/6â€"Î²-Catenin Signaling in Mechanotransduction. *Front. Endocrinol. (Lausanne).* **5**, (2015).
32. Ai, M., Heeger, S., Bartels, C. F., Schelling, D. K. & Osteoporosis-Pseudoglioma Collaborative Group. Clinical and Molecular Findings in Osteoporosis-Pseudoglioma Syndrome. *Am. J. Hum. Genet.* **77**, 741–753 (2005).
33. Teebi, A. S., Al-Awadi, S. A., Marafie, M. J., Bushnaq, R. A. & Satyanath, S. Osteoporosis-pseudoglioma syndrome with congenital heart disease: A new association. *J. Med. Genet.* **25**, 32–36 (1988).
34. Leupin, O. *et al.* Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. *J. Biol. Chem.* **286**, 19489–19500 (2011).
35. Barik, A. *et al.* LRP4 is critical for neuromuscular junction maintenance. *J. Neurosci.* **34**, 13892–13905 (2014).
36. Kariminejad, A. *et al.* Severe Cenani-Lenz syndrome caused by loss of LRP4 function. *Am. J. Med. Genet. Part A* **161**, 1475–1479 (2013).
37. Ohkawara, B. *et al.* LRP4 third β-propeller domain mutations cause novel congenital myasthenia by compromising agrin-mediated MuSK signaling in a position-specific manner. *Hum. Mol. Genet.* **23**, 1856–1868 (2014).
38. Morris, J. A. *et al.* An atlas of genetic influences on osteoporosis in humans and mice. *Nat. Genet.* **51**, 258–266 (2019).
39. Wuttke, M. *et al.* A catalog of genetic loci associated with kidney function from analyses of a million individuals. *Nat. Genet.* **51**, 957–972 (2019).

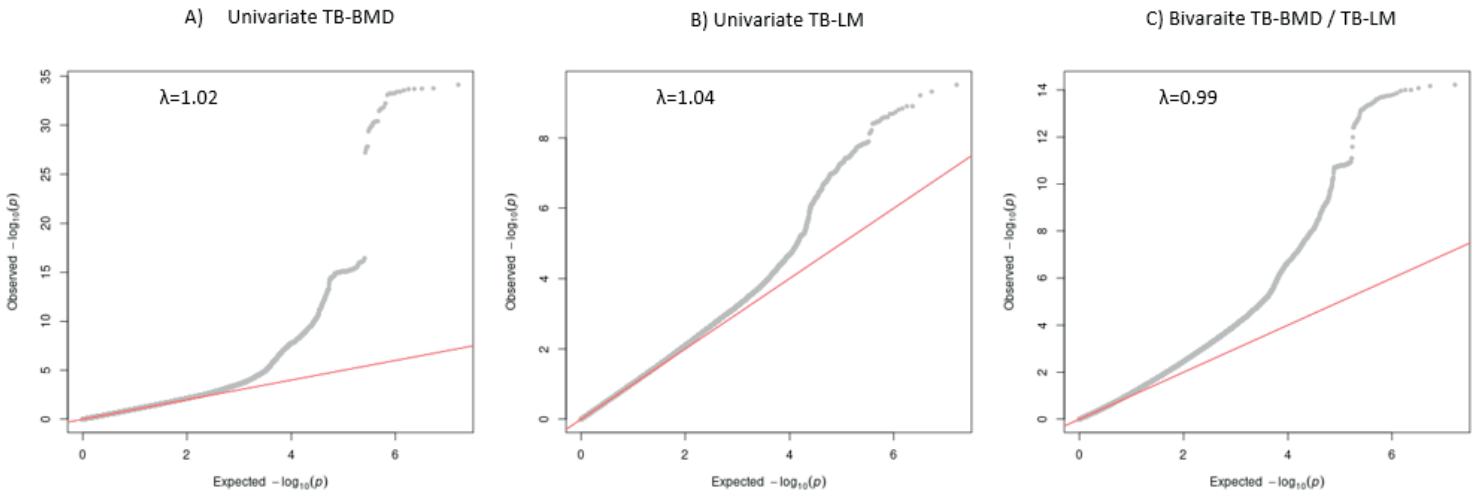
40. Savage, J. E. *et al.* Genome-wide association meta-analysis in 269,867 individuals identifies new genetic and functional links to intelligence. *Nat. Genet.* **50**, 912–919 (2018).
41. Turcot, V. *et al.* Protein-altering variants associated with body mass index implicate pathways that control energy intake and expenditure in obesity. *Nat. Genet.* **50**, 26–35 (2018).
42. #1, Y.-F. P. *et al.* Genome-wide association study of appendicular lean mass in UK Biobank cohort 1. doi:10.1101/643536
43. Barefield, D. Y. *et al.* Experimental Modeling Supports a Role for MyBP-HL as a Novel Myofilament Component in Arrhythmia and Dilated Cardiomyopathy. *Circulation* **136**, 1477–1491 (2017).
44. Lahm, H. *et al.* Myosin binding protein H-like (MYBPHL): a promising biomarker to predict atrial damage. *Sci. Rep.* **9**, (2019).
45. Lee, S.-J. *et al.* Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 18117–22 (2005).
46. DiGirolamo, D. J., Singhal, V., Chang, X., Lee, S.-J. & Germain-Lee, E. L. Administration of soluble activin receptor 2B increases bone and muscle mass in a mouse model of osteogenesis imperfecta. *Bone Res.* **3**, 14042 (2015).
47. Puolakkainen, T. *et al.* Treatment with soluble activin type IIB-receptor improves bone mass and strength in a mouse model of Duchenne muscular dystrophy. *BMC Musculoskelet. Disord.* **18**, 20 (2017).
48. Goh, B. C. *et al.* Activin receptor type 2A (ACVR2A) functions directly in osteoblasts as a negative regulator of bone mass. *J. Biol. Chem.* **292**, 13809–13822 (2017).
49. Scholtysek, C. *et al.* PPARβ/δ governs Wnt signaling and bone turnover. *Nat. Med.* **19**, 608–613 (2013).
50. Burri, L., Thoresen, G. H. & Berge, R. K. The role of PPAR activation in liver and muscle. *PPAR Research* (2010). doi:10.1155/2010/542359
51. Kazanskaya, O. *et al.* The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* **135**, 3655–3664 (2008).
52. Cambier, L., Plate, M., Sucov, H. M. & Pashmforoush, M. Nkx2-5 regulates cardiac growth through modulation of Wnt signaling by R-spondin3. *Dev.* **141**, 2959–2971 (2014).
53. Chen, D. *et al.* Dentin dysplasia type I—A dental disease with genetic heterogeneity. *Oral Diseases* **25**, 439–446 (2019).
54. Lotta, L. A. *et al.* Association of Genetic Variants Related to Gluteofemoral vs Abdominal Fat Distribution with Type 2 Diabetes, Coronary Disease, and Cardiovascular Risk Factors. *JAMA - J. Am. Med. Assoc.* **320**, 2553–2563 (2018).
55. Spranger, J., Winterpacht, A. & Zabel, B. The type II collagenopathies: A spectrum of chondrodysplasias. *European Journal of Pediatrics* **153**, 56–65 (1994).
56. Graham, S. E. *et al.* Sex-specific and pleiotropic effects underlying kidney function identified from GWAS meta-analysis. *Nat. Commun.* **10**, (2019).
57. Kichaev, G. *et al.* Leveraging Polygenic Functional Enrichment to Improve GWAS Power. *Am. J. Hum. Genet.* **104**, 65–75 (2019).
58. O'Seaghdha, C. M. *et al.* Meta-Analysis of Genome-Wide Association Studies Identifies Six New Loci for Serum Calcium Concentrations. *PLoS Genet.* **9**, e1003796 (2013).
59. Sussman, M. A. Developing hearts need their SPEG. *Circulation* **119**, 213–4 (2009).
60. Wang, H. *et al.* Insights from genotype–phenotype correlations by novel SPEG mutations causing centronuclear myopathy. *Neuromuscul. Disord.* **27**, 836–842 (2017).

61. Huntoon, V. *et al.* SPEG-deficient skeletal muscles exhibit abnormal triad and defective calcium handling. *Hum. Mol. Genet.* **27**, 1608–1617 (2018).
62. Miyatake, S. *et al.* Biallelic Mutations in MYPN, Encoding Myopalladin, Are Associated with Childhood-Onset, Slowly Progressive Nemaline Myopathy. *Am. J. Hum. Genet.* **100**, 169–178 (2017).
63. Bang, M. L. *et al.* Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. *J. Cell Biol.* **153**, 413–427 (2001).
64. Ma, K. & Wang, K. Interaction of nebulin SH3 domain with titin PEVK and myopalladin: implications for the signaling and assembly role of titin and nebulin. *FEBS Lett.* **532**, 273–278 (2002).

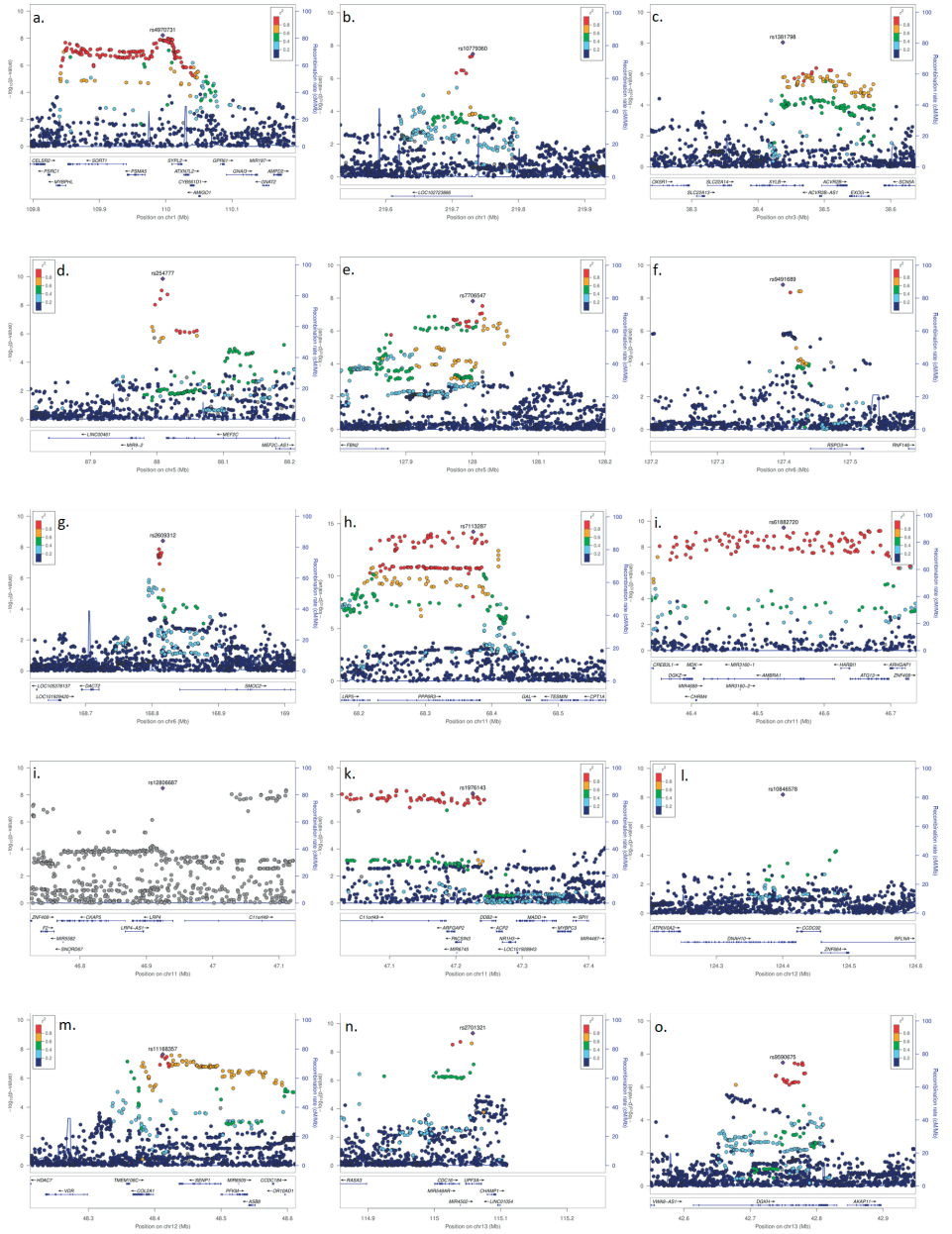
SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Significant loci from the univariate TB-BMD and TB-LM GWAS.

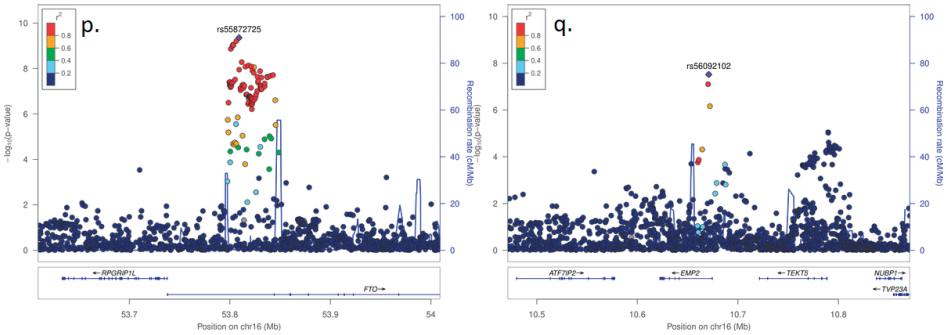
Trait	Locus	SNP	BP	EA	NEA	EAF	Effect	se	P	Closest Gene
TB-BMD	1p36.12	rs10493013	22703035	T	C	0.82	-0.09	0.01	$2.20x10^{-15}$	*WNT4*
	1p31.3	rs2566752	68656697	T	C	0.62	-0.06	0.01	$4.64x10^{-12}$	*WLS*
	2q14.2	rs141795717	119539208	A	G	0.02	0.21	0.03	$7.73x10^{-11}$	*EN1*
	2q24.3	rs6719426	166585566	A	G	0.48	-0.05	0.01	$7.08x10^{-11}$	*CSRNP3 / GALNT3*
	2q37.1	rs7584554	234308782	T	G	0.44	-0.05	0.01	$3.36x10^{-8}$	*DGKD*
	3p22.1	rs7609599	41164756	A	G	0.46	-0.05	0.01	$7.74x10^{-9}$	*CTNNB1*
	6q22.33	rs4580892	127409882	T	C	0.29	0.05	0.01	$4.39x10^{-8}$	*RSPO3*
	7q21.3	rs7787512	96131103	T	C	0.65	0.06	0.01	$1.42x10^{-12}$	*C7orf76/ SHFM1*
	7q31.31	rs2908004	120969769	A	G	0.46	0.10	0.01	$7.31x10^{-35}$	*CPED1/WNT16*
	8q24.12	rs7014574	119977077	T	C	0.56	-0.05	0.01	$4.41x10^{-10}$	*TNFRSF11B*
	10q21.1	rs7902708	54439728	C	G	0.11	-0.07	0.01	$2.96x10^{-8}$	*MBL2*
	11p14.1	rs6416055	27281007	T	C	0.63	-0.05	0.01	$4.20x10^{-8}$	*CCDC34*
	11p11.2	rs79232684	47171714	A	G	0.09	0.09	0.01	$4.86x10^{-9}$	*C11orf49*
	11q13.2	rs60212556	68305494	T	C	0.27	-0.08	0.01	$8.11x10^{-17}$	*PPP6R3*
	11q14.2	rs618926	86867252	C	G	0.68	0.05	0.01	$1.19x10^{-8}$	*TMEM135*
	12q13.13	rs10747668	53737461	T	C	0.71	-0.06	0.01	$7.08x10^{-10}$	*SP7*
	13q14.11	rs9533094	42965837	A	G	0.54	0.05	0.01	$1.08x10^{-8}$	*FABP3P2*
	17q21.31	rs66838809	41798621	A	G	0.08	0.09	0.02	$1.72x10^{-9}$	*MEOX1/DUSP3*
TB-LM	1q41	rs1810144	219649771	T	G	0.30	-0.06	0.01	$4.84x10^{-10}$	*ZC3H11B*
	2q35	rs1810144	220350452	T	C	0.66	0.05	0.01	$6.19x10^{-10}$	*SPEG*
	10q21.3	rs12415105	69958631	A	G	0.49	-0.04	0.01	$3.69x10^{-8}$	*MYPN*
	16q12.2	rs56094641	53806453	A	G	0.60	-0.05	0.01	$3.69x10^{-8}$	*FTO*



Supplementary Figure1 | QQ plots for the genome-wide meta-analysis for A) TB-BMD; B) TB-LM and C) Bivariate meta-analysis

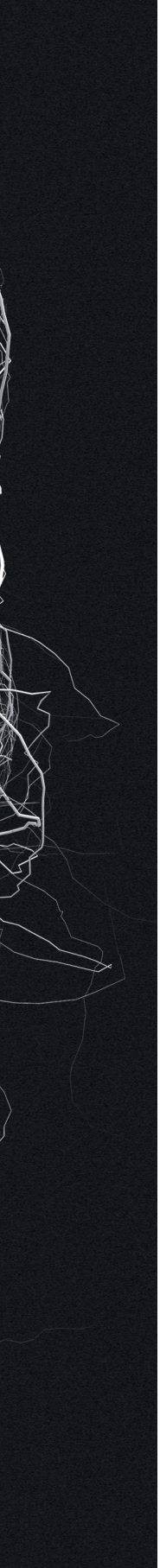


Supplementary Figure 2 | Regional association plots for each of the genome-wide significant loci 1p13.3 (a), 1q41(b), 3p22.2 (c), 5q14.3 (d), 5q23.3 (e), 6q22.33 (f), 6q27 (g),11q13.2 (h),11p11.2 (i, j, k),12q24.31 (l),12q13.11 (m),13q34 (n),13q14.11 (o),16q12.2 (p), 16p13.13 (q)



Supplementary Figure2 | Regional association plots for each of the genome-wide significant loci 1p13.3 (a), 1q41(b), 3p22.2 (c), 5q14.3 (d), 5q23.3 (e), 6q22.33 (f), 6q27 (g),11q13.2 (h),11p11.2 (i, j, k),12q24.31 (l),12q13.11 (m),13q34 (n),13q14.11 (o),16q12.2 (p), 16p13.13 (q) *(continued)*





4.3

Genetic correlations: falls, muscle, bone and fracture

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*Denotes equal supervision

In press

ABSTRACT

Both extrinsic and intrinsic factors predispose older people to fall. We performed a genome-wide association analysis to investigate how much of an individual's fall susceptibility can be attributed to genetics in 89,076 cases and 362,103 controls from the UK Biobank Study. The analysis revealed a small, but significant SNP-based heritability (2.7%) and identified three fall-associated loci ($P \leq 5x10^{-8}$). Polygenic risk scores in two independent settings showed patterns of polygenic inheritance. Risk of falling had positive genetic correlations with fracture risk, identifying for the first time a pathway independent of bone mineral density. There were also positive genetic correlations with insomnia, neuroticism, depressive symptoms, attention deficit hyperactivity disorders, and different medication traits. Negative genetic correlations were identified with muscle strength, intelligence and subjective well-being. Brain, and in particular cerebellum tissue, showed the highest gene expression enrichment for fall-associated variants. Overall, despite the highly heterogenic nature underlying fall risk, a proportion of the susceptibility can be attributed to genetics.

INTRODUCTION

Falls are a growing healthcare problem in older adults. They are a major contributor to immobility and premature nursing home placement[1]. Furthermore, they are a leading cause of unintentional injuries, which require medical treatment[2], and increase the demand on healthcare resources. At present, between 0.85-1.50% of the total health care expenditures in Europe, North America, and Australia are fall-related costs[3]. As the global population continues to grow and become older, healthcare costs related to falls will grow accordingly[4].

There are numerous extrinsic and intrinsic factors predisposing older adults to fall that have been intensively studied in the past decades[5,6,7]. A number of the intrinsic ones, particularly postural balance, gait speed, muscle function and cognition have a recognized heritable component[8,9,10], suggesting that investigation into the genetic influence on falls may be warranted. Pharmacogenetic variability may also contribute to drug-induced falls as a result on the variability of drug responses and risk of adverse effects of medications[11,12]. Twins studies have found that familial factors, consisting of genetic and shared environmental influences, explain about 35% of the variability in the likelihood of experiencing at least one incident fall and 45% of the variability in the risk for recurrent falls[13]. Genetic variation is stable across the human lifespan and identification of genetic factors for falling may help optimizing effective fall prevention programs i.e., improved fall risk stratification; while also, providing biological insight into their etiology. So far, few studies have been performed to identify genetic factors underlying fall risk, likely due to lack of a well-powered discovery setting. In a candidate-gene study without replication (N_{cases}=955; N_{total}=4,163), Judson *et al* [14] reported that female carriers of the *ACTN3* genetic variant (rs1815739), which is associated with reduced muscle mass and force, had 33% higher risk of falling compared to non-carriers. However, to date, no genome-wide associations studies (GWAS) have been performed to identify genetic variants associated with increased fall risk in a hypothesis-free context.

To better understand the genetic architecture of falls, we undertook the first large-scale GWAS to determine the heritability of fall risk, identify genetic variants associated with falling, their underlying biology, and to investigate the relationship with fall-associated conditions and traits.

RESULTS

Genome-wide Association Study of Falling

Our study included data from 451,179 (89,076 cases) white European individuals (40-69 years) from the UK Biobank. We tested 7,745,390 million variants (minor allele frequency (MAF) >0.01, imputation quality >0.3) for association with fall risk. LD-score regression showed no sign of genomic inflation (Intercept=1.01) compatible with a polygenic architecture of the trait and no evidence for stratification. We identified two loci associated with fall risk mapping to 7p21.3 near *PER4* (rs2709062-A, OR=1.03, $P=3.4x10^{-8}$) (**Figure 1A**) and 19q12 near *TSHZ3* (rs2111530-G, OR=1.03, $P=1.2x10^{-8}$) (**Figure 1B**). Moreover, 58 SNPs were associated at $P<5x10^{-6}$ of which 15 were associated at genome-wide suggestive level (sGWAS, $P<5.0x10^{-7}$) (**Table 1, Supplementary Figure 1**). LD-score regression showed no sign of genomic inflation (Intercept=1.01) compatible with a polygenic architecture of the trait and no evidence for stratification (**Supplementary Figure 2**). Using a genome-wide gene-based approach, implemented by MAGMA, we identified nine fall associated genes (**Supplementary Figure 3**); the MAGMA gene-set results were later served as an input for the tissue expression analysis. The majority of the SNPs with $P<5x10^{-6}$ (**Supplementary Figure 4A**) were located in intergenic or intronic regions, and >70% of the variants overlapped chromatin state annotations (**Supplementary Figure 4B**) of potential involvement in gene regulation however, only 3.3% (regulomeDB score≤2) possessed strong regulatory potential (**Supplementary Figure 4C**). The 7p21.3 risk locus did not harbor genes with relevant eQTL and/or chromatin interactions (**Supplementary Figure 5A**). The *TSHZ3* gene at the 19q12 locus was annotated by eQTLs in the thyroid tissue and was also implicated by chromatin interactions in the mesendodermal tissue and the mesenchymal stem cells (**Supplementary Figure 5B**). However, none of the lead SNPs showed any evidence for eQTL effects. Finally, the lead SNP on locus 5q21.2 was previously associated with variety of traits such as insomnia, depression and neurotism. In addition, several suggestive SNPs were associated with body composition measures such as BMI, fat mass and fat free mass (**Supplementary Table 1**).

Replication

We followed for replication the 17 suggestive GWS SNPs ($P<5.0x10^{-7}$) from the discovery sample in two smaller and older prospective population-based studies, namely the Rotterdam Study (1,009 cases and 4,925 controls) and B-PROOF (1,206 cases and 1,364 controls) cohorts. The B-PROOF Study is a clinical trial on B-vitamin supplements in older adults of advanced age (mean age 74.1±6.5 years) in which fall risk was assessed using retrospective questionnaires at baseline and prospective fall calendars; while the Rotterdam Study is a population-based cohort with fall infor-

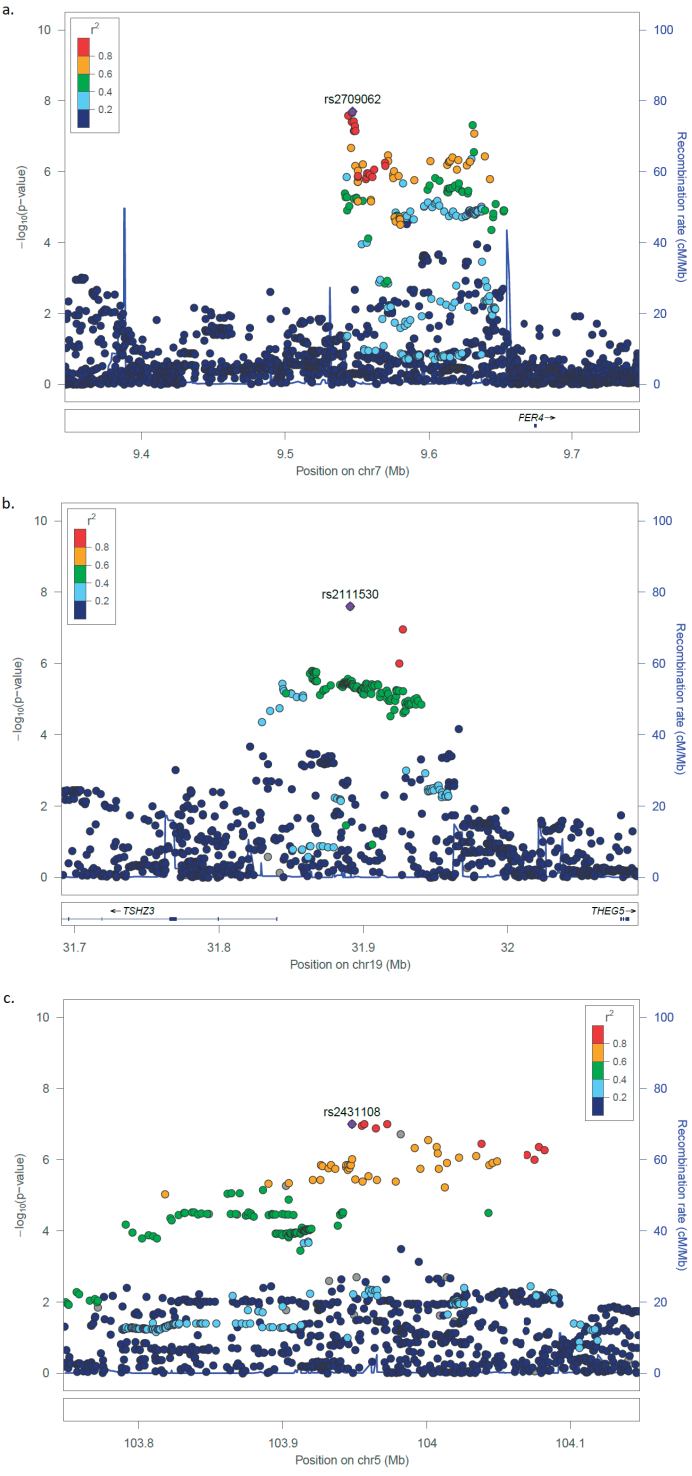


Figure 1 | Regional association plots displaying 7p21.3 (A), 19q12 (B) and 5q21.2 (C) loci. In each plot, the $-\log_{10}$ of p values are on the left y-axis; the SNP genomic position (hg19) on the x-axis; the estimated recombination rate from 1000 genomes (March 2015 EUR) are on the right y-axis and plotted in blue. SNPs are colored red to reflect linkage disequilibrium (LD) with the most significant SNP in purple (pairwise r^2 from 1000 genomes March 2014 EUR).

Table 1 | Lead SNPs of loci associated with increased risk of falling ($p<5x10^{-7}$)

Locus	Annotation	Closest gene	Position	SNP	EA	NEA	EAF	UKBB N=89,076/362,103		Rotterdam Study N= 1,009/4,925		B-PROOF N=1,206/1,364		Combined N=91,219/368,392	
								OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	p
Genome-wide significant loci ($P\leq5x10^{-8}$)															
5q21.2		*RP11-6N13.1*	103947968	rs2431108	C	T	0.33	1.03 (1.02-1.04)	$9.9x10^{-8}$	1.13 (1.03-1.25)	0.01	0.98 (0.88-1.10)	0.77	1.03 (1.02-1.04)	$4.20x10^{-8}$
7p21.3	intergenic	*PER4*	9546806	rs2709062	A	G	0.50	1.03 (1.02-1.04)	$2.4x10^{-8}$	1.08 (0.98-1.19)	0.12	1.13 (1.01-1.26)	0.03	1.03 (1.02-1.04)	$4.04x10^{-9}$
19q12	intergenic	*TSHZ3*	31891006	rs2111530	G	A	0.39	1.03 (1.02-1.04)	$2.5x10^{-8}$	1.00 (0.91-1.11)	0.88	1.08 (0.86-1.18)	0.20	1.03 (1.02-1.04)	$1.82x10^{-8}$
Genome-wide suggestive loci ($P<5x10^{-7}$)															
1p13.3	intergenic	*NTNG1*	107666942	rs76259395	A	G	0.03	1.03 (1.02-1.05)	$6.6x10^{-8}$	1.03 (0.90-1.19)	0.64	1.02 (0.82-1.26)	0.86	1.03 (1.02-1.05)	$6.46x10^{-8}$
1p13.2	intronic	*FAM212B*	112274162	rs6658723	T	C	0.02	1.02 (1.01-1.03)	$2.6x10^{-7}$	1.00 (0.91-1.11)	0.96	1.01 (0.90-1.11)	0.93	1.02 (1.01-1.03)	$3.14x10^{-7}$
2p16.1	intergenic	*EIF3FP3*	59295476	rs67174662	A	G	0.02	1.02 (1.01-1.03)	$5.0x10^{-7}$	0.99 (0.90-1.10)	0.91	1.02 (0.91-1.14)	0.77	1.02 (1.01-1.03)	$5.56x10^{-7}$
2p16.1	intergenic	*BCL11A*	60333030	rs974135	T	C	0.33	1.02 (1.02-1.03)	$3.9x10^{-7}$	0.96 (0.87-1.07)	0.51	0.94 (0.83-1.06)	0.34	1.02 (1.01-1.03)	$9.45x10^{-7}$
3p14.2	intronic	*FHIT*	60138226	rs7616516	A	G	0.07	1.05 (1.03-1.06)	$2.3x10^{-7}$	0.95 (0.79-1.14)	0.56	0.99 (0.85-1.16)	0.92	1.04 (1.03-1.06)	$3.49x10^{-7}$
5p35.2	intergenic	*DRD1*	174888896	rs2471020	C	T	0.59	1.02 (1.01-1.03)	$4.8x10^{-7}$	1.07 (0.97-1.17)	0.20	1.03 (0.92-1.16)	0.61	1.02 (1.01-1.03)	$2.43x10^{-7}$
6p21.1	intronic	*TRERF1*	42360455	rs72857666	T	C	0.03	1.07 (1.04-1.10)	$1.5x10^{-7}$	0.83 (0.61-1.14)	0.26	0.96 (0.68-1.36)	0.81	1.07 (1.04-1.09)	$2.85x10^{-7}$
7p21.3	intergenic	*NXPH1*	9629549	rs12666565	C	T	0.18	1.03 (1.02-1.04)	$4.7x10^{-7}$	1.06 (0.93-1.21)	0.41	1.13 (0.98-1.31)	0.10	1.03 (1.02-1.04)	$1.77x10^{-7}$

Table 1 | Lead SNPs of loci associated with increased risk of falling ($p<5x10^{-7}$) (*continued*)

Locus	Annotation	Closest gene	Position	SNP	EA	NEA	EAF	UKBB		Rotterdam Study		B-PROOF		Combined	
								N=89,076/362,103		N= 1,009/4,925		N=1,206/1,364		N=91,219/368,392	
								OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	p
11p14.1	intergenic	*BDNF*	27643725	rs11030084	C	T	0.03	1.03 (1.02-1.04)	$1.1x10^{-7}$	1.10 (0.96-1.25)	0.16	0.96 (0.91-1.20)	0.55	1.03 (1.02-1.04)	$8.92x10^{-8}$
11p14.1	intronic	*MPPED2*	30492581	rs494221	A	G	0.03	1.03 (1.02-1.04)	$6.7x10^{-8}$	0.98 (0.88-1.08)	0.68	0.88 (0.78-0.98)	0.02	1.02 (1.01-1.03)	$3.01x10^{-7}$
11p15.5	intronic	*TSPAN4*	855372	rs28672671	A	G	0.03	1.03 (1.02-1.04)	$5.1x10^{-8}$	0.95 (0.83-1.07)	0.27	0.89 (0.78-1.01)	0.08	1.03 (1.02-1.04)	$2.23x10^{-7}$
14.q21.2	intergenic	*RPL10L*	46984874	rs12884871	C	T	0.03	1.03 (1.02-1.04)	$1.4x10^{-7}$	1.05 (0.94-1.14)	0.37	0.97 (0.86-1.09)	0.59	1.03 (1.02-1.04)	$1.44x10^{-7}$
19q12		*ZNF536*	30772256	rs28633123	T	C	0.21	1.03 (1.02-1.04)	$9.4x10^{-8}$	1.65 (0.93-1.20)	0.37	1.00 (0.87-1.16)	0.96	1.03 (1.02-1.04)	$7.29x10^{-7}$
20q11.23	intronic	*CTNNBL1*	36382855	rs6063547	G	T	0.03	1.03 (1.02-1.04)	$6.8x10^{-8}$	1.06 (0.92-1.21)	0.44	0.99 (0.85-1.16)	0.92	1.03 (1.02-1.04)	$6.45x10^{-8}$

Footnote: lead SNP is defined as SNP with the lowest p-value. EA=effect allele; NEA: non-effect allele; EAF=effect allele frequency; OR= odds ratio; CI= confidence interval; p= p value of the SNP-falls association.

mation from participants (mean age 69.5±9.2 years) collected retrospectively from baseline questionnaires. We considered loci to replicate if they passed the nominal (P<0.05) significant threshold in the replication effort or the GWS (P<5x10^{-8}) in the combined meta-analysis. The top two SNPs from the discovery phase remained GWS significant in the combined meta-analysis, which also brought one additional locus (5q21.3) mapping to *RP11-6N13.1* above the GWS threshold (**Table 1; Figure 1C**). The new locus did not harbour genes with relevant eQTL and/or chromatin interactions (**Supplementary Figure 5C**).

Polygenic Risk Scores

Next, we evaluated the ability of polygenic risk scores (PRSs) constructed from the UK Biobank GWAS results to discriminate between fallers and non-fallers in two independent prospective cohorts. We hypothesized falling risk to follow a polygenic mode inheritance, i.e., is influenced by numerous genes with small individual effects and, hence, non-GWS SNPs may also contribute to the genetic component of falling risk. Therefore, PRS were constructed using PRSice[15] for a series of P-value thresholds ranging from 5x10^{-8} to 1. Variants (P<0.05, MAF>0.05 and imputation quality>0.3) were clumped before analysis (r^2 < 0.1, window: 300Kb) to obtain the most significant SNP in the locus. In line with polygenic inheritance, the PRS explained a small, but robust, proportion of the trait variance (max R^2=0.29%) along different P-value thresholds. In the BPROOF Study, the PRS derived from GWS variants (P≤ 5x10^{-8}) was associated with prospective falls (reported by fall calendar) and explained the largest fraction of the trait variance (max R^2=0.29%) (**Figure 2A**). In contrast, the PRSs constructed from variants in the lower significance thresholds (P ≤ 5x10^{-3}) were the ones more strongly associated with retrospectively collected falls (reported by baseline questionnaires) (**Figure 2B and 2C**).

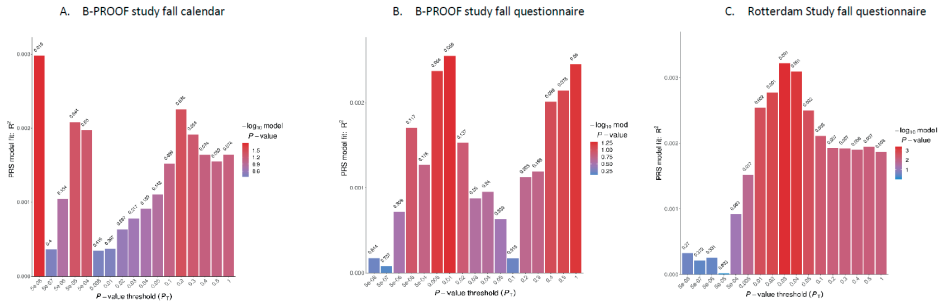


Figure 2 | Association of falls PRS adjusted for age and sex across several different p-value thresholds (x-axis) within two different populations (A. B-PROOF study falls calendar; B. B-PROOF Study falls questionnaire and C. Rotterdam Study falls questionnaire). The number on top of the bars represent p values of the association between the score and fall risk. Color scale: -log$_{10}$ of P-value.

Individual and Shared Heritability of Falls

We then used LD-Score Regression (LDSR) to estimate the heritability (individual and shared) between falls and different diseases and traits[16]; We considered traits that are closely phenotypically related with fall risk such as musculoskeletal, neurological, psychological ones and use of a variety of medications. As expected, the SNP-based heritability of falls was low (h2=0.027, SE=0.002). In relation to other traits (**Figure 3**), falling had positive genetic correlation with fracture (r_g=0.45, SE=0.05) and was negatively genetically correlated (r_g=-0.17, SE=0.04) with muscle strength. Of particular note, there was no evidence of any genetic correlation between fall risk with bone mineral density or lean mass. We also used LDSR to explore whether falls are genetically correlated with a range of neurological, psychiatric and behavioral traits (**Figure 3**). Falls were strongly positively correlated with insomnia, neuroticism, depressive symptoms and attention deficit hyperactivity disorders (ADHD). We observed a small to moderate negative correlation with intelligence/IQ (r_g=-0.12, SE=0.04) and subjective well-being (r_g=-0.29, SE=0.05) (**Figure 3**). Medication use is a well-established risk factor for falls, either directly (affecting balance, attention or muscle tone) or indirectly (as proxies of underlying conditions influencing the risk of falling; joint pain, arthrosis, cardiovascular diseases among many others). A recent GWAS in approximately 320,000 individuals from the UK Biobank[17], identified 505 independent genetic loci associated with medication use grouped across 23 categories. Using this

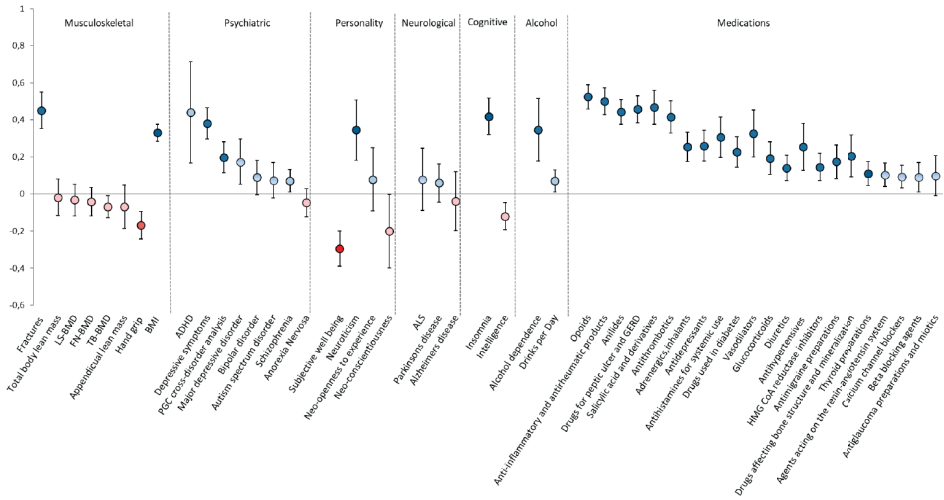


Figure 3 | Estimates of the genetic correlation between falls and different traits and medications. The bars around the point estimates represent confidence intervals. The blue colored point estimates indicate a positive whereas the red colored point estimates a negative genetic correlation. The lighter shades are indication of non-significant correlation after correcting for multiple testing (p=0.05/49=0.001).

data, we observed positive genetic correlations between fall risk and use of medication such as opioids, anti-inflammatory and anti-rheumatic drugs, anilids and drugs for peptic ulcer and gastro-esophageal reflux disease.

Enrichment of Gene Expression Across Tissues

Further, to quantify the enrichment of the falls-associated SNPs signals across different tissues we used an extension of LDSR, namely *Stratified Linkage Disequilibrium Score Regression* (LDSC-SEG) and identified significant enrichment confined to tissues from the central nervous system and particularly those derived from the cerebellum (**Figure 4A**). We then used *Generalized gene-set analysis of GWAS data* (as implemented in *MAGMA*[18]) and also identified significant enrichment of the signals confined to gene

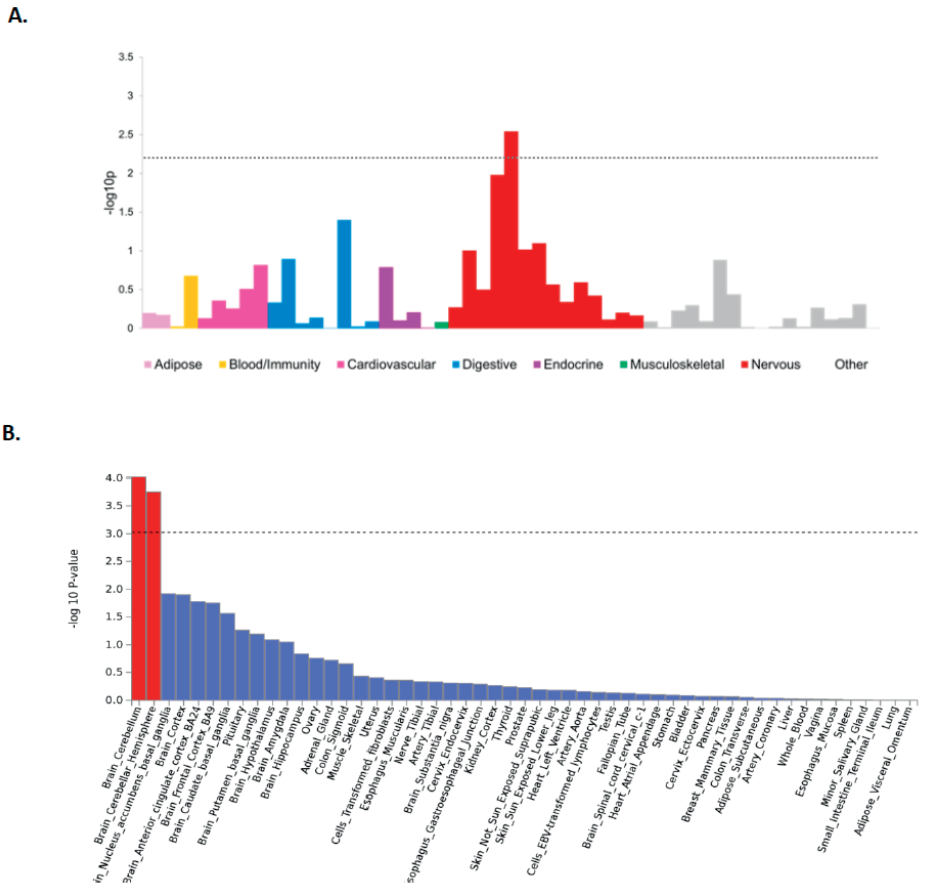


Figure 4 | GTEx brain tissues were positively enriched for falls-associated variants. A. Heritability partitioning enrichment estimates across tissue groups using LD-SEG; B. Tissue enrichment analysis using MAGMA. The most enriched brain tissue is the cerebellum

expression in cerebellar tissue (**Figure 4B**). The latter findings indicate that biological processes related to movement control of limbs, locomotion, adaptation of posture and dynamic regulation of balance originated at the cerebellum could play a role shaping the complex mechanisms underlying fall risk.

Mendelian Randomization

We then tested whether the association between seven risk factors and falls was causal by using genetic factors as instruments within a two-sample Mendelian Randomization approach. Although there are many determinants and conditions that influence the risk of falling, well-powered GWASs allowing the use of adequate genetic instruments were available for *alcohol consumption*[19], *alcohol dependence*[20], *body mass index* (*BMI*)[21], and *relative hand grip strength* defined as the average of measurements of right and left hand divided by weight [22]. In addition, we evaluated the effect of antihypertensive medication use on fall risk using genetic variants mapping to target genes for several antihypertensive drugs. For this purpose we used genetic instruments for antihypertensive drug use that were recently created by Gill et al[23]. These antihypertensive drug medications showed no significant evidence for a causal effect. Alcohol dependence was nominally associated with increased fall risk (OR=1.04, 95%CI=1.01 to 1.08, P=0.03) but not after Bonferroni correction. Alcohol consumption also showed no significant evidence for a causal effect on falls. We did find evidence for a causal effect of BMI (OR=1.13, 95%CI=1.06 to 1.20, P<0.0001) and relative hand grip strength (OR=0.41, 95%CI=0.23 to 0.41,P<0.0001) on fall risk (**Supplementary Table 2**), suggesting that interventions targeted at improving muscle function and weight control may be successful at decreasing falling risk.

DISCUSSION

To our knowledge, this is the first GWAS for fall risk performed to date. Our findings indicate that fall risk is an extremely heterogeneous polygenic trait with large environmental influence. Despite such complex genetic architecture, we were able to identify variants in three loci mapping to chromosomes 5q21.2, 7p21.3 and 19q12. Polygenic risk scores were associated with falling risk in two independent population-based settings. On aggregate, associated markers show significant enrichment for genes expressed in cerebellar tissue providing insight into potential mechanisms mediating fall risk. Shared genetic variation with fracture risk, muscle strength, medication use and other risk factors suggests potential pleiotropic relationships and common biological pathways could mediate diverse aspects of falling risk.

Overall, falls are multifactorial in origin and many different pathways can contribute to the individual propensity to fall. Given the polygenic nature of fall risk, it is expected that a large number of genes influence the risk of falling, each with a very small contribution. Therefore, large-scale analysis such as the present UK Biobank study, are required to discover such real, but weak genetic associations. Additionally, the low heritability of fall risk in our study indicates that there is a strong environmental component underlying the risk of falling. On the other hand, in twins studies 35% of the variability in the likelihood of at least one incident fall and 45% of the variability in the risk for recurrent falls was attributed to genetic factors[13]. Notably, it has been shown that heritability may not be constant during the lifespan[24], with heritability typically decreasing with increasing age as a consequence of accumulation of environmental influences with aging[25].

We demonstrated that risk for falling has a strong positive genetic correlation with fracture and low grip strength, while no significant correlation was observed with BMD or lean mass. This finding implicates a mechanistic pathway influencing fracture risk that is independent of bone mineral density. Fracture occurs when the force applied to a bone is greater than the overall bone strength. Low BMD is a key component of bone fragility, i.e. a necessary but not sufficient cause of fractures [26]. Although falls may be an independent predictor of fractures, the fall-related fracture risk dramatically increases in presence of low BMD. However, low BMD alone explains less than one-half of all non-vertebral fractures[27,28] and fractures occurring at higher BMD thresholds require the presence of other risk factors. Recent studies have postulated that falls and not osteoporosis per se constitute the strongest risk factor for fractures in extremely old individuals[29,30]. Therefore, falling may be a major contributing factor to overall fracture occurrence independent of, and in addition to age and BMD[31]. The strong genetic correlation of falls with fractures but not BMD corroborates the findings of epidemiological studies, while it also provides novel insights into the complex interplay between these traits.

The GWAS signal on chromosome 19q12 (rs2111530; MAF=0.39) maps in the vicinity (50.6 kb) of *TSHZ3*, gene encoding a zinc-finger transcription factor involved in diverse developmental processes. Recently, a GWAS meta-analysis found a variant (rs6510186) near this gene associated with total body bone mineral density (TB-BMD) exclusively in middle-aged adults (45-60 years old)[32]. Nevertheless, this variant is unlikely to arise from the same association signal, as it is not in LD with the top variant from our GWAS (distance 236.4 Kb, r^2=0.003). There is scarce information about the function of the *TSHZ3* gene, except that it is suggested to be part of a node of 24 genes with high degree of connectivity (i.e. hub genes) with strong levels of expression in early fetal cerebral cortical development[33,34]. Reduced expression of *TSHZ3* resulting in caspase upregulation has been proposed to be involved in the pathogen-

esis of Alzheimer's disease[35]. Moreover, genetic linkage[36,37] and association studies[38] have identified this gene as a potential candidate for autism susceptibility disorders. Altogether, this could suggest a role of *TSHZ3* in cortical development and in the pathogenesis of neurodevelopmental disorders. Pending additional evidence of its involvement in cerebellar biology, our findings suggest its plausible involvement in susceptibility to fall. Annotations relevant to *TSHZ3* include both eQTLs and chromatin interactions which could further support evidence for the gene involvement in falling susceptibility. The 7p21.3 variant (rs2709062; MAF=0.50) is also intergenic located 127.1 Kb upstream of *PER4*, a pseudogene, affiliated with lncRNA and of which little is known. Next, the combined meta-analysis of all participating cohorts yielded another signal (rs2431108 MAF=0.33) surpassing the GWS threshold. The SNP maps to 5q21.2 in RP11-6N13.1, a long intervening/intergenic noncoding RNA (lincRNA), which does not overlap protein-coding genes. This SNP have been previously reported in association with several psychiatric traits such as insomnia, anxiety, neurotism, and depression. Overall, none of the lead SNPs show any evidence for significant eQTLs and given the lack of information we cannot claim if the closes genes are also the causal genes.

The polygenic risk score analyses performed in two independent prospective cohorts corroborated the polygenic architecture underlying fall risk. The joint effect of PRS could reliably determine some of the variation underlying fall risk, implicating variants associated up to a significance level of $5x10^{-3}$. The scores constructed from the two most significantly associated variants were more strongly associated when using the more sensitive prospective falling assessment (fall calendars, used in the B-PROOF study); while employing a retrospective definition of falling (as used in the discovery and in both replication studies) resulted in the strongest polygenic risk scores arising from the inclusion of variants below the genome-wide significant level. These discrepancies can be the consequence of many different factors, including differences in assessment methodology, as retrospective falls were self-reported and may underestimate the occurrence of falls as a result of recall bias[39]. On the other hand, fall calendars are considered the best tool available for reliable fall assessment in older adults, providing more accurate information on falls (i.e., prospective assessment) as participants report fall incidents each week. Nevertheless, the PRS results should be interpreted with caution given the low SNP heritability of fall risk[40].

Medication use is recognized as an important risk factor for falling, while polypharmacy among the elderly has increased dramatically in the past decades[41]. Epidemiologically, several types of psychotropic, cardiac and analgesic drugs are associated with a significant risk of falling, typically including sedatives and hypnotics such as benzodiazepines, antipsychotics, antidepressants, diuretics, antiepileptics and opioids[42,43,44]. In line with the epidemiological relationship, we found that falls had significant genetic correlation with use of most of these medication categories and in

the expected directions. The strongest genetic correlation was observed with opioids and anti-inflammatory drug use. These findings suggest that some of the genetic predisposition for falling risk is shared with use of medication associated with falling risk. Yet, the causal mechanistic pathways can differ across different pleiotropic (vertical vs horizontal) relationships. Vertical pleiotropy will be relevant for medications causally-related to fall risk, where the genetic correlation with a condition will also be related to the drug indication all together, pointing to a common biological pathway influencing all three components (fall risk, condition and medication). Another form of vertical pleiotropy will be expected to arise when the medication is not necessarily causally related to fall risk (e.g. NSAIDs), but the condition driving the drug indication (e.g. arthrosis or other musculoskeletal disorder affecting mobility) will be the causal factor leading to falling. True horizontal pleiotropy is much more difficult to ascertain in a largely polygenic trait[45] like falling risk, but we can expect it to arise with specific conditions through biological pathways influencing neurological/cerebellar function as further discussed below.

Reduced hand grip strength and increased BMI are risk factors identified by MR to be causally related to fall risk. A decreased in relative handgrip strength was observed to be causally associated with increased risk of falling. Therefore, muscle weakness is a clinically relevant risk factor for falls that should be assessed and treated in older adults at risk for falls[46]. Our MR results also support the evidence from recent observational studies that older obese individuals have greater risk of falling[47,48]. The exact mechanisms by which BMI increases the risk of falling in older adults remain unclear and require further exploration. One possible explanation is that obesity may alter balance control which is an important risk factor for falling[49].

Further, using grouped cell-type and tissue expression analysis, the cerebellum was the most significant enriched tissue. The cerebellum plays an important role in motor control and maintaining postural balance[50]. Cerebellar disorders can lead to orthostatic hypotension, vertigo and syncope, all important risk factors for falls[51,52]. All these factors can increase the fall risk, either individually or combined.

Some limitations of our study need to be noted. Although self-reported measures can appear robust for other phenotypes such as birthweight[53], it is possible that recall bias may have influenced the assessment of fall risk. Falling is a complex, heterogeneous trait and most of the cases it may be attributed to non-genetic factors (e.g., medication use, mobility disorders, and hazardous household environments). Also, given the UKBB age range (40-69 years) and inclusion of European ancestry individuals only, we cannot assume that our results generalize to other age groups in whom falls are more common, and/or ancestral populations. There are other factors explaining the large heterogeneity underlying fall risk. Different fall patterns are observed between young and old people; while on the other hand, the number

of co-morbid conditions and medication use associated with falls also increase with age[54]. The genetic susceptibility for falls may be of less importance in individuals with multiple fall risk factors regardless of age. Different factors can increase the risk of falling across different age decades. Next, the heritability and genetic susceptibility of fall risk might be higher in individuals with recurrent falls which we were not able to test in the current study. Similarly, a GWAS on medication use-related falls is warranted to address the causal role of medication and understand further the biological pathways underlying fall risk. Finally, participants from the UK Biobank were included in the GWASs of both relative handgrip strength and falls. This sample overlap might increase the probability of a Type I error resulting in false-positive findings; thus, it needs to be replicated in independent efforts. There is an ongoing collection of hospital admission data in the UK Biobank that may provide an improved and well-powered resource for future research. Lastly, in our GWAS study we only tested SNPs for association with falling risk. We were not able to take into account other genetic structural variations such as single number variations (CNVs) or inversions which may also contribute to the genetic landscape of falling risk. Similarly, we also did not test for potential epigenetic modifications.

In conclusion, our study demonstrated that fall risk is a heritable, heterogeneous and polygenic trait genetically correlated with fracture risk and grip strength, among other neuropsychiatric and medication traits. The cerebellum tissue enrichment of falls-associated variants supports the mediation of postural balance in the etiology of falls. Our study provides novel biological insight that can be used for optimizing strategies directed at preventing falls and their associated deleterious consequences in aging individuals.

SUBJECTS AND METHODS

Study Population

Our analyses were performed using data from the UK Biobank study. Briefly, the UK Biobank is a large prospective cohort study of approximately a half-million adult (ages 40-69) participants living in the United Kingdom (UK), recruited from 22 centers across the UK in 2006-2010[55]. We use a subsample of the total study who were identified as or white European ancestry using a combination of genetic principle components and self-reported ethnicity. Ethical approval was granted by the Northwest Multi-centre Research Ethics Committee, and written informed consent was obtained from all participants.

Assessment of Falls

The number of falls in the UK Biobank was self-reported via a touch screen questionnaire. In total 89,076 individuals have reported that they have had one or more falls answering to the question "In the last year have you had any falls?". Individuals who selected "prefer not to answer" or "do not know" were set to missing; the rest of the population were classified as controls (N=362,103).

GWAS Data and Imputation

The majority of UK Biobank participants were genotyped with the Affymetrix UK Biobank Axiom Array (Santa Clara, CA, USA), while 10% of participants were genotyped with the Affymetrix UK BiLEVE Axiom Array. Detailed quality control and imputation procedures are described elsewhere[56]. Imputation was performed using the Haplotype Reference Consortium panel[57]. Only participants of white European-ancestry (identified using a k-means clustering approach based on genetic principal components, as well as self-identification) were analyzed.

Association Analysis

Genetic association analyses were performed using BOLT-LMM[58]. Briefly, this method uses a linear mixed model to account for relatedness and population structure using a relationship matrix. Fall risk was corrected for age and sex in logistic regression models. SNP association was tested for all autosomal variants. Individuals were excluded based on unusually high heterozygosity or >5% missing genotype rate, a mismatch between self-reported and genetically-inferred sex. SNP exclusions were made based on, low minor allele frequency (<1%) and low imputation quality (info<0.3). SNPs with $P \leq 5 \times 10^{-7}$ were considered suggestive while SNPs with $P \leq 5 \times 10^{-8}$ were considered genome wide significant (GWS).

Replication and Meta-analysis

The suggestive SNPs ($P \leq 5 \times 10^{-7}$) were later followed for replication in smaller and older prospective population-based studies i.e. the Rotterdam Study (1,009 cases and 4,925 controls) and B-PROOF (1,206 cases and 1,364 controls) cohorts. The Rotterdam Study is an ongoing population bases cohort within a suburb in Rotterdam. Its design, objective and methods have been described in detail[59]. Briefly, the study was initiated in 1989 and 7,983 participants aged 55 and above were included. Participants were interviewed and underwent an extensive set of examination that were repeated every 4-5 years. B-PROOF has been also described in details elsewhere[60]. In short, it is a multi-center, randomized, placebo-controlled, double-blinded trial investigating the efficacy of vitamin B and folic acid supplementation on the prevention of fractures in people aged 65 and older. In total 2,919 participants were included and followed for

2-3 years. Both studies have been approved by the medical ethics committee. In the Rotterdam Study, fall history was assessed from baseline questionnaire. In B-PROOF, retrospective and prospective falls were reported. Prevalent falls were assessed using a baseline questionnaire while fall incidences during follow-up were reported prospectively using a falls' calendar in a period of 2-3 years. The baseline questionnaire in both cohorts consisted of a single question: "*Have you fallen in the past 12 months?*". In B-PROOF we used fall incidence for the GWAS analysis as it provided larger sample size (500 participants had missing information on prevalent falls). Both studies used commercially available genome-wide arrays to genotyped their participants. SNPs were imputed to the Haplotype Reference Consortium (HRC) reference panel[57] (build 37) using the Michigan Imputation Server (MIS).

Gene-based Testing and Functional Mapping

Gene-based GWAS analysis was carried out with MAGMA 1.6[18] using the default settings implemented in FUMA[61]. According to the number of tested genes, the level of gene-wide significance was set at $0.05/18{,}615=2.7\times10^{-6}$. Functional annotation (i.e. prioritization, annotation and interpretation) of GWAS results was also performed using FUMA[61].

Polygenic Risk Scores (PRSs)

We used imputed genotype data from the B-PROOF and the Rotterdam Study cohorts to calculate PRSs for each participant using the PRsice software[15]. For the construction of the PRS we first selected SNPs with $P<0.05$, MAF>0.05 and imputation quality>0.3 from the UK Biobank GWAS. Next, we excluded SNPs with MAF<0.05 and imputation quality<0.3 in the Rotterdam study and B-PROOF. In total 436,130 SNPs passed these thresholds and were followed in the PRS analysis. PRSs were created for a series of *P*-value thresholds ranging from 5×10^{-8} to 1. In total, 17 PRSs were created. SNPs were pruned with PLINK (version 1.9) using stringent clumping thresholds based on both linkage-disequilibrium and distance using an r^2 of 0.1 and a distance of 300 Kb. We tested each of the 17 PRSs in relation to fall risk adjusted for age and sex stratified by cohort. In the B-PROOF study we tested the scores with both prevalent and incident falls. We reported the proportion of variance explained (based on R^2) by each fall PRS.

Linkage Disequilibrium Score Regression

SNP-based heritability and genomic inflation

To estimate the genomic inflation in the data and the SNP-based heritability of falls, we used LD-score regression (LDSR)[62]. The LD-score regression intercept provides estimate of inflation due to population stratification or model misspecification; im-

portantly, it is not unduly affected by polygenicity[63]. We used pre-calculated LD-score from the 1000 Genomes European reference population (https://data.broadinstitute.org/alkesgroup/LDSCORE/).

Shared genetic architecture of falls and other traits

To estimate the genetic correlation between falls and other complex traits and diseases, we used (cross-trait) LD-score regression[16] as implemented in the online web utility LDHub[64]. This method uses the cross-products of summary test statistics from two GWASs and regresses them against a measure of how much variation each SNP tags (its LD-score)[65]. The data base of the LDHub web utility contains a range of data relating to genetic effects on common diseases and phenotypes. In addition, locally we estimated the genetic correlation between fall risk and 22 medication classes from the latest GWAS on medication use [17]; where medications were self-reported and classified using the Anatomical Therapeutic Chemical (ACT) Classification System into 1,752 categories (with minimum 10 users) after careful evaluation of the medication record data.

Stratified heritability and functional enrichment and tissue specificity analyses of falls-associated variants

To identify tissues and cell types that are likely to be involved in falling susceptibility, we applied LD-score regression to specifically expressed genes (LDSC-SEG)[66]. Annotation data was obtained from the LD-score website (https://github.com/bulik/ldsc). For each tissue, we ranked genes by a t-statistic for differential expression, using sex and age as covariates and excluding all samples in related tissues[66]. For example, we compared expression in cerebellum samples to expression in all non-brain samples. We used the top 10% of genes by this ranking and used stratified LD-score regression to estimate the contribution of genomic annotations to per-SNP falls heritability, adjusting for 24 main annotations categories (eg. coding, UTR, promotor, intron) in the baseline model[67]. In addition, we performed MAGMA Tissue Expression analysis (using FUMA) to test relationships between tissue specific gene expression and disease-gene associations. MAGMA was performed using the result of gene-set analysis (gene-based P-value) and tested for one side (greater) with conditioning on average expression across all tissue types.

Mendelian Randomization

We used the largest previously published GWAS meta-analyses of the trait included in the MR analysis. The construction of genetic instruments for antihypertensive drug targets are explained in details by Gill et al [23]. To reduce potential bias due to population stratification, we restricted the analyses to studies with participants of

European descent. In addition, instrumental variables which were nominally ($p<0.05$) associated with fall risk were excluded from the analysis. The resulting individual SNP effect estimates were pooled using inverse-variance weighted (IVW) meta-analysis. We applied a conservative Bonferroni corrected threshold to account for the multiple testing (that is, $\alpha=0.007$, because seven exposures were assessed). To test the third assumption (a lack of pleiotropic effects of the SNPs on the outcome, independent of the exposure), we used Mendelian randomization-Egger regression. Moreover, as sensitivity analyses for robust causal inference, we additionally performed Mendelian randomization analyses using a weighted median estimator. The analyses were conducted with the R-package MendelianRandomization [68].

REFERENCES

1. Rubenstein, L. Z. CLINICAL RISK ASSESSMENT, INTERVENTIONS AND SERVICES Falls in older people: epidemiology, risk factors and strategies for prevention Background and epidemiology. *Age Ageing* 35–2 (2006). doi:10.1093/ageing/afl084
2. Verma, S. K. *et al.* Falls and Fall-Related Injuries among Community-Dwelling Adults in the United States. *PLoS One* **11**, e0150939 (2016).
3. Heinrich, S., Rapp, K., Rissmann, U., Becker, C. & König, H.-H. Cost of falls in old age: a systematic review. *Osteoporos. Int.* **21**, 891–902 (2010).
4. Hartholt, K. A. *et al.* Costs of falls in an ageing population: A nationwide study from the Netherlands (2007–2009). *Injury* **43**, 1199–1203 (2012).
5. Tinetti, M. E., Speechley, M. & Ginter, S. F. Risk Factors for Falls among Elderly Persons Living in the Community. *N. Engl. J. Med.* **319**, 1701–1707 (1988).
6. Graafmans, W. C. *et al.* Falls in the elderly: a prospective study of risk factors and risk profiles. *Am. J. Epidemiol.* **143**, 1129–36 (1996).
7. Gale, C. R., Cooper, C. & Aihie Sayer, A. Prevalence and risk factors for falls in older men and women: The English Longitudinal Study of Ageing. *Age Ageing* **45**, 789–794 (2016).
8. Wagner, H., Melhus, H., Pedersen, N. L. & Michaëlsson, K. Heritability of impaired balance: a nationwide cohort study in twins. *Osteoporos. Int.* **20**, 577–583 (2009).
9. Ortega-Alonso, A. *et al.* A twin study on the heritability of walking ability among older women. *J. Gerontol. A. Biol. Sci. Med. Sci.* **61**, 1082–5 (2006).
10. Reed, T., Fabsitz, R. R., Selby, J. V & Carmelli, D. Genetic influences and grip strength norms in the NHLBI twin study males aged 59-69. *Ann. Hum. Biol.* **18**, 425–32
11. Just, K. S., Schneider, K. L., Schurig, M., Stingl, J. C. & Brockmöller, J. Falls: the adverse drug reaction of the elderly and the impact of pharmacogenetics. *Pharmacogenomics* **18**, 1281–1297 (2017).
12. Ham, A. C. *et al.* CYP2C9 Genotypes Modify Benzodiazepine-Related Fall Risk: Original Results From Three Studies With Meta-Analysis. *J. Am. Med. Dir. Assoc.* **18**, 88.e1-88.e15 (2017).
13. Pajala, S. *et al.* Genetic Factors and Susceptibility to Falls in Older Women. *J. Am. Geriatr. Soc.* **54**, 613–618 (2006).
14. Judson, R. N. *et al.* The Functional ACTN3 577X Variant Increases the Risk of Falling in Older Females: Results From Two Large Independent Cohort Studies. *Journals Gerontol. Ser. A* **66A**, 130–135 (2011).
15. Euesden, J., Lewis, C. M. & O'Reilly, P. F. PRSice: Polygenic Risk Score software. *Bioinformatics* **31**, 1466–1468 (2015).
16. Bulik-Sullivan, B. *et al.* An atlas of genetic correlations across human diseases and traits. *Nat. Genet.* **47**, 1236–1241 (2015).
17. Wu, Y. *et al.* Genome-wide association study of medication-use and associated disease in the UK Biobank. *Nat. Commun.* **10**, 1891 (2019).
18. de Leeuw, C. A., Mooij, J. M., Heskes, T. & Posthuma, D. MAGMA: Generalized Gene-Set Analysis of GWAS Data. *PLOS Comput. Biol.* **11**, e1004219 (2015).
19. Liu, M. *et al.* Association studies of up to 1.2 million individuals yield new insights into the genetic etiology of tobacco and alcohol use. *Nat. Genet.* **51**, 237–244 (2019).
20. Walters, R. K. *et al.* Transancestral GWAS of alcohol dependence reveals common genetic underpinnings with psychiatric disorders. *Nat. Neurosci.* **21**, 1656–1669 (2018).

21. Locke, A. E. *et al.* Genetic studies of body mass index yield new insights for obesity biology. *Nature* **518**, 197–206 (2015).
22. Tikkanen, E. *et al.* Biological Insights Into Muscular Strength: Genetic Findings in the UK Biobank. *Sci. Rep.* **8**, 6451 (2018).
23. Gill, D. *et al.* Use of Genetic Variants Related to Antihypertensive Drugs to Inform on Efficacy and Side Effects. *Circulation* **140**, 270–279 (2019).
24. Steves, C. J., Spector, T. D. & Jackson, S. H. D. Ageing, genes, environment and epigenetics: what twin studies tell us now, and in the future. *Age Ageing* **41**, 581–586 (2012).
25. Reynolds, C. A. *et al.* Quantitative Genetic Analysis of Latent Growth Curve Models of Cognitive Abilities in Adulthood. *Dev. Psychol.* **41**, 3–16 (2005).
26. Trajanoska, K. *et al.* Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study. *BMJ* **362**, k3225 (2018).
27. Stone, K. L. *et al.* BMD at Multiple Sites and Risk of Fracture of Multiple Types: Long-Term Results From the Study of Osteoporotic Fractures. *J. Bone Miner. Res.* **18**, 1947–1954 (2003).
28. Trajanoska, K. *et al.* Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. *Bone* **114**, 116–124 (2018).
29. Masud, T. & Morris, R. O. Epidemiology of falls. *Age Ageing* **30 Suppl 4**, 3–7 (2001).
30. Järvinen, T. L. N., Sievänen, H., Khan, K. M., Heinonen, A. & Kannus, P. Shifting the focus in fracture prevention from osteoporosis to falls. *BMJ* **336**, 124–126 (2008).
31. Geusens, P. *et al.* The relationship among history of falls, osteoporosis, and fractures in postmenopausal women. *Arch. Phys. Med. Rehabil.* **83**, 903–906 (2002).
32. Medina-Gomez, C. *et al.* Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects. *Am. J. Hum. Genet.* **102**, 88–102 (2018).
33. Caubit, X. *et al.* TSHZ3 deletion causes an autism syndrome and defects in cortical projection neurons. *Nat. Genet.* **48**, 1359–1369 (2016).
34. Kang, H. J. *et al.* Spatio-temporal transcriptome of the human brain. *Nature* **478**, 483–489 (2011).
35. Kajiwara, Y. *et al.* FE65 Binds Teashirt, Inhibiting Expression of the Primate-Specific Caspase-4. *PLoS One* **4**, e5071 (2009).
36. A full genome screen for autism with evidence for linkage to a region on chromosome 7q. International Molecular Genetic Study of Autism Consortium. *Hum. Mol. Genet.* **7**, 571–8 (1998).
37. Liu, J. *et al.* A genomewide screen for autism susceptibility loci. *Am. J. Hum. Genet.* **69**, 327–40 (2001).
38. Hussman, J. P. *et al.* A noise-reduction GWAS analysis implicates altered regulation of neurite outgrowth and guidance in autism. *Mol. Autism* **2**, 1 (2011).
39. Ganz, D. A., Higashi, T. & Rubenstein, L. Z. Monitoring Falls in Cohort Studies of Community-Dwelling Older People: Effect of the Recall Interval. *J. Am. Geriatr. Soc.* **53**, 2190–2194 (2005).
40. Choi, S. W., Mak, T. S. H. & O'Reilly, P. A guide to performing Polygenic Risk Score analyses. *bioRxiv* 416545 (2018). doi:10.1101/416545
41. Charlesworth, C. J., Smit, E., Lee, D. S. H., Alramadhan, F. & Odden, M. C. Polypharmacy Among Adults Aged 65 Years and Older in the United States: 1988-2010. *J. Gerontol. A. Biol. Sci. Med. Sci.* **70**, 989–95 (2015).

42. de Vries, M. *et al.* Fall-Risk-Increasing Drugs: A Systematic Review and Meta-Analysis: I. Cardiovascular Drugs. *J. Am. Med. Dir. Assoc.* **19**, 371.e1-371.e9 (2018).
43. Seppala, L. J. *et al.* Fall-Risk-Increasing Drugs: A Systematic Review and Meta-Analysis: II. Psychotropics. *J. Am. Med. Dir. Assoc.* **19**, 371.e11-371.e17 (2018).
44. Seppala, L. J. *et al.* Fall-Risk-Increasing Drugs: A Systematic Review and Meta-analysis: III. Others. *J. Am. Med. Dir. Assoc.* **19**, 372.e1-372.e8 (2018).
45. Khera, A. V. *et al.* Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. *Nat. Genet.* **50**, 1219–1224 (2018).
46. Moreland, J. D., Richardson, J. A., Goldsmith, C. H. & Clase, C. M. Muscle Weakness and Falls in Older Adults: A Systematic Review and Meta-Analysis. *J. Am. Geriatr. Soc.* **52**, 1121–1129 (2004).
47. Fjeldstad, C., Fjeldstad, A. S., Acree, L. S., Nickel, K. J. & Gardner, A. W. The influence of obesity on falls and quality of life. *Dyn. Med.* **7**, 4 (2008).
48. Mitchell, R. J., Lord, S. R., Harvey, L. A. & Close, J. C. T. Associations between obesity and overweight and fall risk, health status and quality of life in older people. *Aust. N. Z. J. Public Health* **38**, 13–18 (2014).
49. Dutil, M. *et al.* The impact of obesity on balance control in community-dwelling older women. *Age (Dordr).* **35**, 883–90 (2013).
50. Takakusaki, K. Functional Neuroanatomy for Posture and Gait Control. *J. Mov. Disord.* **10**, 1–17 (2017).
51. Gangavati, A. *et al.* Hypertension, orthostatic hypotension, and the risk of falls in a community-dwelling elderly population: the maintenance of balance, independent living, intellect, and zest in the elderly of Boston study. *J. Am. Geriatr. Soc.* **59**, 383–9 (2011).
52. Schlick, C. *et al.* Falls and fear of falling in vertigo and balance disorders: A controlled cross-sectional study. *J. Vestib. Res.* **25**, 241–251 (2016).
53. Horikoshi, M. *et al.* Genome-wide associations for birth weight and correlations with adult disease. *Nature* **538**, 248–252 (2016).
54. Berry, S. D. & Miller, R. R. Falls: epidemiology, pathophysiology, and relationship to fracture. *Curr. Osteoporos. Rep.* **6**, 149–54 (2008).
55. Sudlow, C. *et al.* UK Biobank: An Open Access Resource for Identifying the Causes of a Wide Range of Complex Diseases of Middle and Old Age. *PLOS Med.* **12**, e1001779 (2015).
56. Welsh, S., Peakman, T., Sheard, S. & Almond, R. Comparison of DNA quantification methodology used in the DNA extraction protocol for the UK Biobank cohort. *BMC Genomics* **18**, 26 (2017).
57. Consortium, the H. R. *et al.* A reference panel of 64,976 haplotypes for genotype imputation. *Nat. Genet.* **48**, 1279–1283 (2016).
58. Loh, P.-R. *et al.* Efficient Bayesian mixed-model analysis increases association power in large cohorts. *Nat. Genet.* **47**, 284–290 (2015).
59. Ikram, M. A. *et al.* The Rotterdam Study: 2018 update on objectives, design and main results. *Eur. J. Epidemiol.* **32**, 807–850 (2017).
60. van Wijngaarden, J. P. *et al.* Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12and folic acid on fracture incidence. *BMC Geriatr.* **11**, 80 (2011).
61. Watanabe, K., Taskesen, E., van Bochoven, A. & Posthuma, D. Functional mapping and annotation of genetic associations with FUMA. *Nat. Commun.* **8**, 1826 (2017).

62. Bulik-Sullivan, B. K. *et al.* LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat. Genet.* **47**, 291–295 (2015).
63. Yang, J. *et al.* Genomic inflation factors under polygenic inheritance. *Eur. J. Hum. Genet.* **19**, 807–12 (2011).
64. Zheng, J. *et al.* LD Hub: a centralized database and web interface to perform LD score regression that maximizes the potential of summary level GWAS data for SNP heritability and genetic correlation analysis. *Bioinformatics* **33**, 272–279 (2017).
65. Kemp, J. P. *et al.* Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis. *Nat. Genet.* **49**, 1468–1475 (2017).
66. Finucane, H. K. *et al.* Heritability enrichment of specifically expressed genes identifies disease-relevant tissues and cell types. *Nat. Genet.* **50**, 621–629 (2018).
67. Finucane, H. K. *et al.* Partitioning heritability by functional annotation using genome-wide association summary statistics. *Nat. Genet.* **47**, 1228–1235 (2015).
68. Yavorska, O. O. & Burgess, S. MendelianRandomization: an R package for performing Mendelian randomization analyses using summarized data. *Int. J. Epidemiol.* **46**, 1734–1739 (2017).

Supplementary Table 1 | Previously reported SNP- and Gene-associations from the GWAS catalog

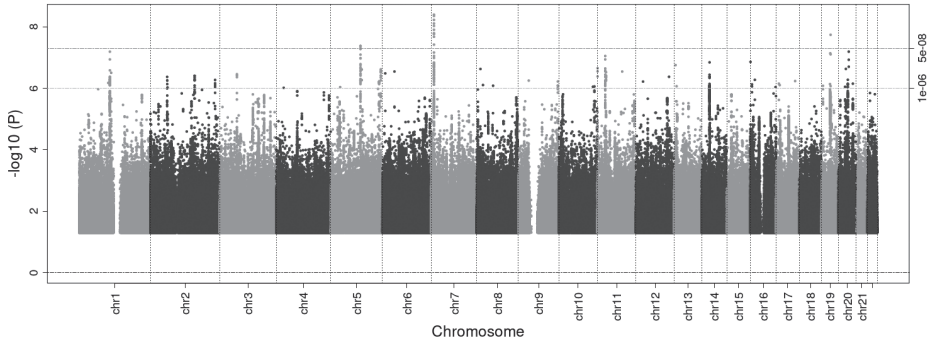
SNP	Know SNP-associations	Gene	Know Gene-associations
rs2431108	Insomnia, Anxiety, Nap during the day, Leg fat percentage, Neurotism, Well-being, Loneliness, Depression, Mornigness	*RP11-6N13.1*	-
rs2709062	-	*PER4*	-
rs2111530	Systolic blood pressure	*TSHZ3*	Lung function, Chronotype measurement, BMD
rs76259395	-	*NTNG1*	Blood proteins, LDL-cholesterol, BMI
rs6658723	BMI, Fat Mass, Waist Circumference, Waist-Hip ratio	*FAM212B*	BMI*
rs67174662	BMI, Fat Mass, Waist Circumference	*EIF3FP3*	Neuroticism, Mood swings
rs974135	Height	*BCL11A*	Blood traits, Education, Cognition
rs7616516	-	*FHIT*	BMI, Physical activity, Smoking, Depression
rs2471020	-	*DRD1*	Eye refractive error
rs72857666	-	*TRERF1*	Blood traits
rs12666565	-	*NXPH1*	chronotype measurement
rs11030084	BMI, Fat Mass, Fat-free Mass, Hip and Waist Circumference, Menarche, Smoking, Risk-taking behaviour	*BDNF-AS*	BMI*, Hip* and Waist circumference, Menarche*, chronotype measurement, smoking*, alcohol consumption
rs494221	Height, Hip circumference, Fat-Free Mass, Basal metabolic rate	*MPPED2*	Estimated glomerular filtration rate, height, Weight circumference, chronotype measurement
rs28672671	-	*TSPAN4*	
rs12884871	-	*RPL10L*	BMI
rs28633123	-	*ZNF536*	Height, BMI, education
rs6063547	-	*CTNNBL1*	-

*the lead SNPs for both traits are in LD

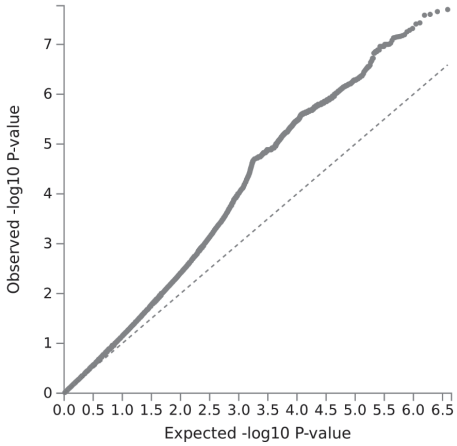
Supplementary Table 2 | Mendelian Randomization analyses of several potential risk factors for falls

	IVW		Weighted median		MR-Egger		Egger Intercept	Number of SNPs
	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	P	
Relative handgrip	0.41 (0.23-0.41)	<0.0001	0.44 (0.23-0.85)	<0.0001	0.24 (0.02-2.09)	0.197	0.63	103
Body mass index	1.13 (1.06-1.20)	<0.0001	1.16 (1.08-1.24)	<0.0001	1.25 (1.08-1.44)	0.002	0.12	77
Alcohol consumption	1.01 (0.99-1.04)	0.38	1.00 (0.97-1.02)	0.82	1.01 (0.94-1.08)	0.84	0.91	97
Alcohol dependence	1.04 (1.01-1.08)	0.029	-		-		-	1
Antihypertensive drugs								
ACE inhibitors	1.00 (0.97-1.03)	0.80	-		-		-	1
Beta-blockers	0.99 (0.97-1.01)	0.47	0.99 (0.98-1.01)	0.37	1.03 (0.96-1.10)	0.45	0.30	6
Calcium channel blockers	1.00 (0.99-1.01)	0.43	0.99 (0.97-1.01)	0.46	0.99 (0.97-1.00)	0.16	0.23	24

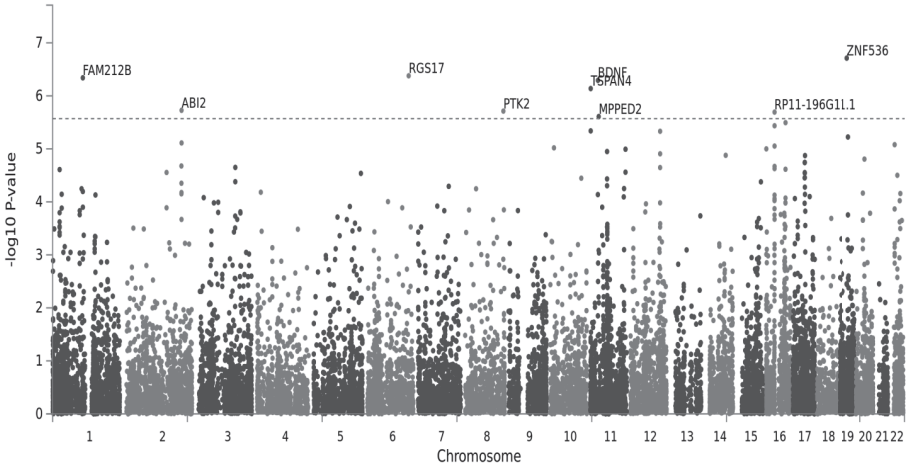
Footnote: The weighted median and the Egger regression MR analyses require at least 3 SNPs for implementation. The OR for relative hand grip (m^2). BMI (kg/m^2) and alcohol consumption (log transformed) are per 1unit increase in the exposure.



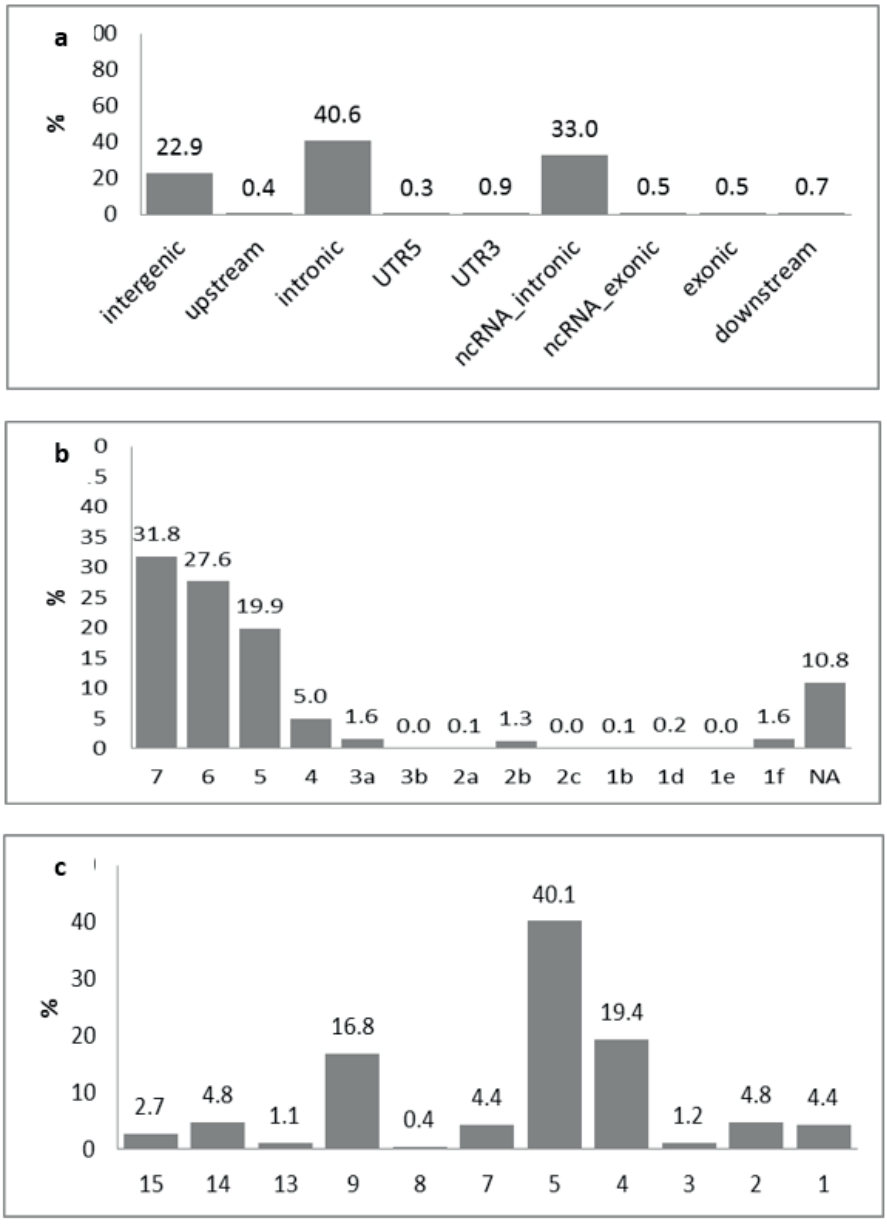
Supplementary Figure 1 | Manhattan Plot of Association Statistics ($-\log_{10}$ (P)) for falling risk for the combined meta-analysis. Each dot represents a SNP and the x axis indicates its chromosomal position (built 37 NCBI). Dashed horizontal red line marks the GWS threshold ($P \leq 5 \times 10^{-8}$).



Supplementary Figure 2 | Quantile-quantile (Q-Q) plot of observed versus expected P values of the GWAS results. The straight line in the Q-Q plot indicates the distribution of SNPs under the null hypothesis

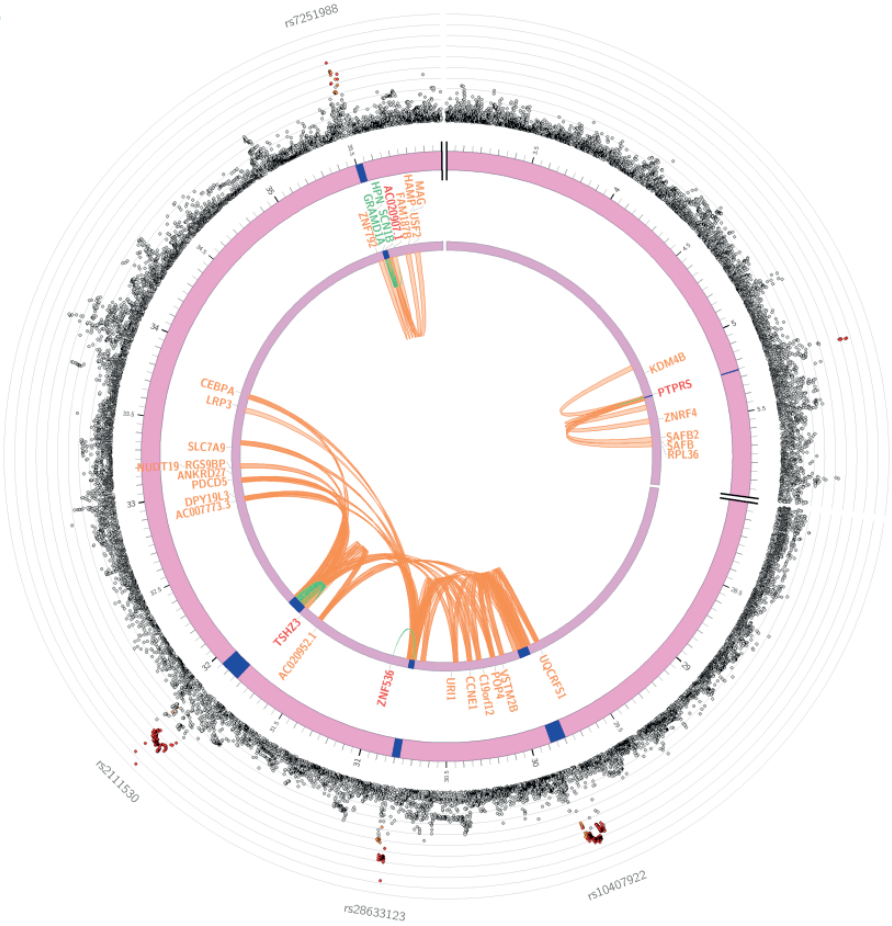


Supplementary Figure 3 | Manhattan plot of the gene-based test as computed by MAGMA. Each dot represents one gene and the x axis indicates its chromosomal position (built 37 NCBI). The dashed red horizontal line marks the gene-number-adjusted threshold (p=0.05/18,185 tested genes).



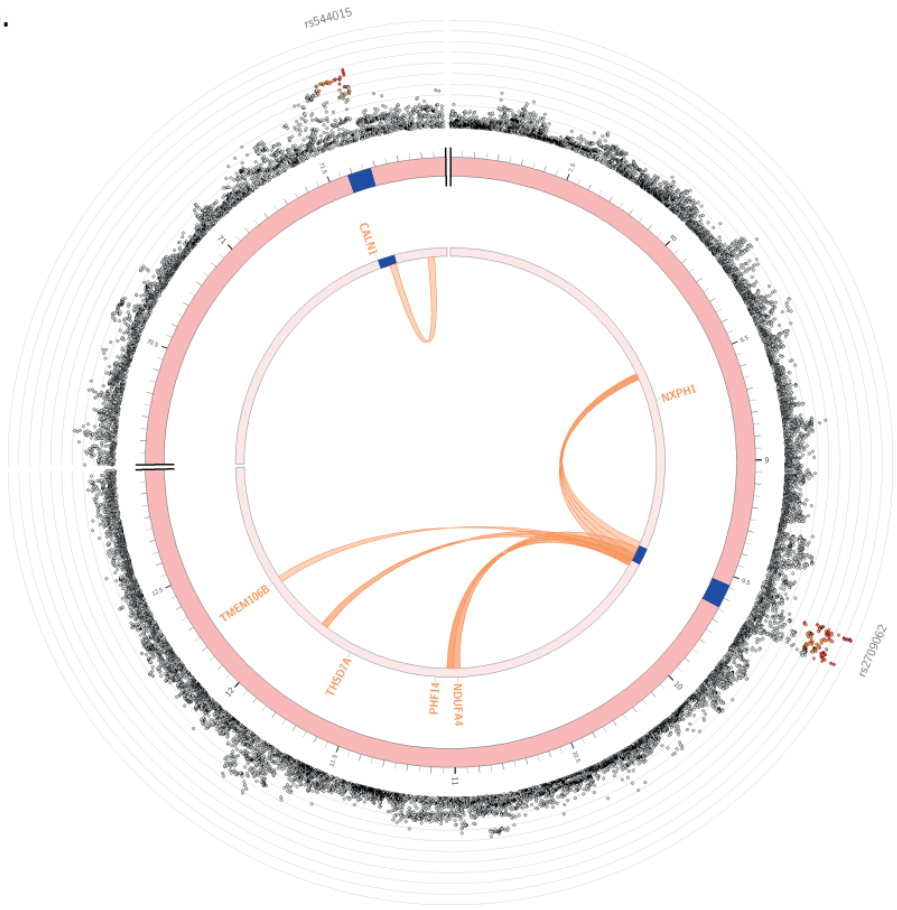
Supplementary Figure 4 | Functional annotation for all SNPs with $r^2 \geq 0.6$ with the top SNPs ($P<1x10^{-6}$). a) Percentage of SNPs according to their functional category; b) Percentage of SNPs according to their RegulomeDB score (x-axis). Lower score indicates a more likely regulatory role; c) Percentage of SNPs according to their minimum chromatin state across 127 tissues. Lower score (x-axis) indicates a more likely regulatory role. NA – not available in RegulomeDB.

a.



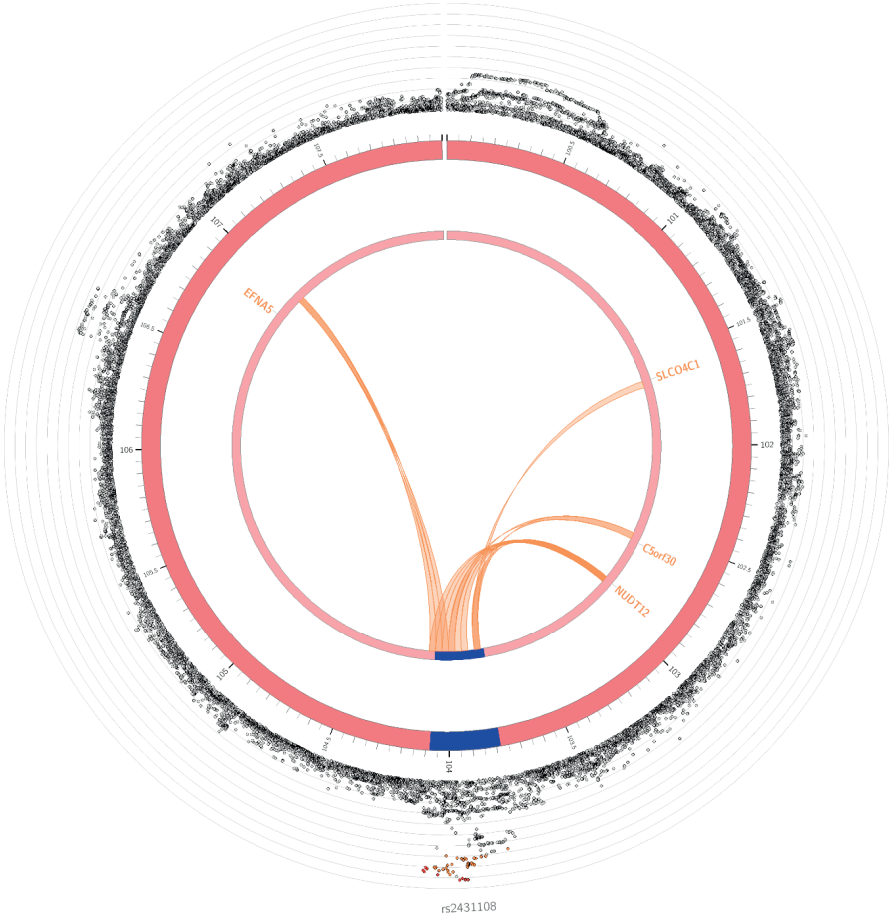
Supplementary Figure 5 | Circos plots demonstrating the results of eQTL and chromatin interaction mapping for loci on chromosome **a)** 7, **b)** 19 and **b)** 5. Genes mapped by Hi-C or eQTLs are colored orange and green, respectively. Genes that were mapped by both eQTL associations and chromatin interaction data are highlighted in red. The highlighted SNPs (blue box) represent the GWS SNPs ($P \leq 5 \times 10^{-8}$).

b.



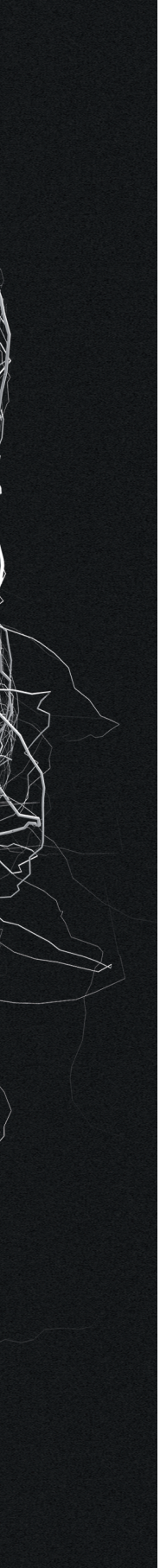
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c.



Supplementary Figure 5 | Circos plots demonstrating the results of eQTL and chromatin interaction mapping for loci on chromosome **a)** 7, **b)** 19 and **b)** 5. Genes mapped by Hi-C or eQTLs are colored orange and green, respectively. Genes that were mapped by both eQTL associations and chromatin interaction data are highlighted in red. The highlighted SNPs (blue box) represent the GWS SNPs ($P \leq 5 \times 10^{-8}$). *(continued)*





4.4

Investigation of shared genetics between osteoarthritis and bone mineral density identifies SMAD3 as a novel osteoarthritis risk locus

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Hum Mol Genet. *2017 Oct 1;26(19):3850-3858.*

ABSTRACT

Osteoarthritis (OA) is a common complex disease with high public health burden and no curative therapy. High bone mineral density (BMD) is associated with an increased risk of developing OA, suggesting a shared underlying biology. Here, we performed the first systematic overlap analysis of OA and BMD on a genome wide scale. We used summary statistics from the GEFOS consortium for lumbar spine (n=31,800) and femoral neck (n = 32,961) BMD, and from the arcOGEN consortium for three OA phenotypes (hip, n_{cases}=3,498; knee, n_{cases}=3,266; hip and/or knee, n_{cases}=7,410; $n_{controls}$=11,009). Performing LD score regression, we found a significant genetic correlation between the combined OA phenotype (hip and/or knee) and lumbar spine BMD (r_g=0.18, P =2.23x10^{-2}), which may be driven by the presence of spinal osteophytes. We identified 143 variants with evidence for cross-phenotype association which we took forward for replication in independent large-scale OA datasets, and subsequent meta-analysis with arcOGEN for a total sample size of up to 23,425 cases and 236,814 controls. We found robustly replicating evidence for association with OA at rs12901071 (OR 1.08 95% CI 1.05–1.11, P_{meta}=3.12x10^{-10}), an intronic variant in the SMAD3 gene, which is known to play a role in bone remodeling and cartilage maintenance. We were able to confirm expression of SMAD3 in intact and degraded cartilage of the knee and hip. Our findings provide the first systematic evaluation of pleiotropy between OA and BMD, highlight genes with biological relevance to both traits, and establish a robust new OA genetic risk locus at SMAD3.

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease of the joints affecting over 40% of people over 70 years.[1] Hallmarks of OA include cartilage degradation, joint-space narrowing, formation of osteophytes within the joint and subchondral bone remodeling.[2,3] Due to a lack of therapeutic options, the main treatment strategy consists of pain management and, in severe cases, joint replacement surgery. [2] OA is a complex disorder with the 18 currently known risk loci accounting for approximately 11% of disease heritability.[4] The biggest OA genome-wide association study (GWAS) published to date was conducted by the arcOGEN consortium in a two-stage design, culminating in a total discovery sample of 7,410 cases and 11,009 controls.[5,6] Cases in the arcOGEN study had radiographic hip or knee OA (defined as a Kellgren Lawrence score 2), and approximately 80% had undergone total joint replacement surgery, indicating disease progression to a severe degree.[6]

In addition to establishing bigger OA sample collections, studying OA in the context of related phenotypes may add to the currently short list of established loci. While most GWAS to date have investigated a single phenotype, it is estimated that around 5% of risk variants and 16% of genes identified through these studies have pleiotropic effects on multiple phenotypes. [7] In recent years, the accumulation of large-scale genomic datasets has made it possible to study genetic pleiotropy by incorporating data from multiple epidemiologically linked traits. Leveraging available summary data of correlated traits to identify common genetic risk factors can help pinpoint causal pathways and refine our understanding of disease mechanisms. OA in itself is a heterogeneous disorder, with heritability varying depending on the affected joint. Of the 18 published risk loci to date, 6 and 7 are associated with knee OA only and hip OA only, respectively, while 5 are associated with both hip and knee OA.[4] This further highlights how phenotypic variation is reflected by genetics, and demonstrates the need for strict phenotype definitions.

The link between bone mineral density (BMD) and OA was first reported in 1972 by Foss and Byers, who observed higher BMD in femoral heads excised during OA-related hip replacement surgery.[8] Since then, a number of cross-sectional and longitudinal studies have found higher femoral neck (FN) and lumbar spine (LS) BMD, as well as total body BMD to be associated with incident OA at the hip, knee and other joint sites.[9-12]

Findings with regards to the relationship between BMD and OA progression are less clear.[13] Elevated bone turnover – usually a marker for decreased BMD - was reported in patients with progressive knee OA compared to patients with stable OA. [14] Decreased baseline femoral neck BMD (FNBMD) has also been associated with knee OA progression.[15,16] Conversely, data from the Rotterdam study showed a non-

significant trend of higher odds of knee OA progression with increased lumbar spine BMD (LSBMD),[17] while another study found no link betweenknee OA progression and total body-or FNBMD.[9]

The largest genetic studies on BMD to date have been carried out by the Genetic Factors for Osteoporosis (GEFOS) consortium: a large-scale GWAS in 2012 found 56 loci associated with BMD;[18] more recently, a rare variant of large effect was identified combining whole-genome sequencing and GWAS imputation.[19] Several biological mechanisms are implicated in both OA and BMD, such as bone remodeling, mesenchymal stem cell differentiation and inflammation.[2,6,18,20] RUNX2, a key transcription factor regulating endochondral ossification and osteoblast differentiation,[21,22] has been associated with both OA and BMD based on its proximity to genome-wide significant variants.[6,18] The other locus with known GWAS hits for both traits is *KLHL42* (or *KLHDC5*), although its biological relevance remains unclear.[6,18]

In addition, Yerges-Armstrong and colleagues have previously shown nominal association of four BMD-linked single nucleotide polymorphisms (SNPs) with knee OA.[23] However, despite the long-established epidemiologic link and shared biology, the genetic overlap of OA and BMD has not yet been assessed on a genome-wide level. Here, we present results from the first genome-wide analysis establishing shared genetic aetiology between OA and BMD.

RESULTS

Known OA Risk Loci in BMD

Six loci associated with OA were nominally significant for FN-or LSBMD, including the *SUPTH3/CDC5L* locus, which is close to *RUNX2*, and *KLHL42/PTHLH* (**Supplementary Material, Table 1**).

Genome-wide Correlation

We used linkage disequilibrium (LD) score regression to get an estimate of the genome-wide genetic correlation between OA and BMD. There was a significant correlation between combined OA and LSBMD (correlation = 0.18; P = 0.022), but not hip or knee OA and LSBMD or between FNBMD and any OA phenotype (**Figure 1**).

Extent of Shared Association Signals

We found evidence for significant overlap of association signals at different P-value thresholds (P_t) between all three OA categories and LSBMD (permutation adjusted P-value (P_{perm})<0.05) (**Table 1**).

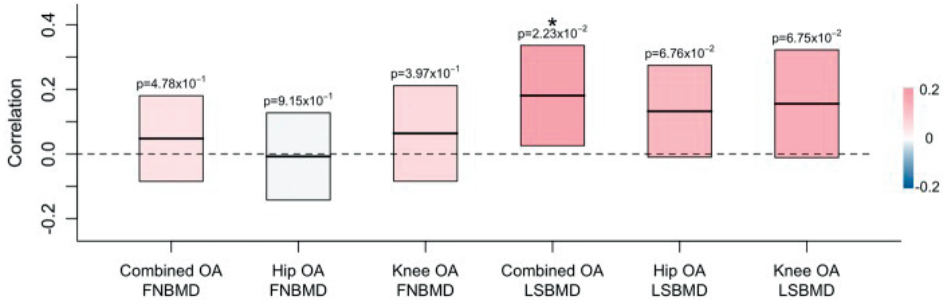


Figure 1 | Genetic correlation between osteoarthritis (OA) and bone mineral density (BMD) as estimated by LD score regression. Rectangles show the correlation estimate (middle horizontal line) and standard errors (upper and lower bounds) of each comparison. Rectangles are coloured according to the strength of correlation. Significant correlation estimates are marked by an asterisk. LSBMD = lumbar spine BMD; FNBMD = femoral neck BMD.

Analysis of the combined OA and LSBMD data resulted in significant overlap P-values at P_t=0.001 and 0.005, as well as at less stringent P_t. Four SNPs overlap at $P_t=5x10^{-4}$ (rs17158899, rs4536164, rs11826287 and rs630765), one of which (rs11826287) is genome-wide significantly associated with FNBMD ($P = 3.61x10^{-14}$) and maps to an intron in *LRP5*.

The highest overlap was observed between hip OA and LSBMD, with six SNPs overlapping at $P_t=5 \times 10^{-4}$ ($P_{perm}=5.7x10^{-5}$). Two of these SNPs are genome-wide significantly associated with BMD in GEFOS (rs1524928, $P = 5.29x10^{-9}$ and rs716255, $P = 2.07x10^{-11}$). A significant overlap was also observed for P_t of 0.001, 0.005 and 0.01 in the hip OA-LSBMD comparison.

Compared to the hip OA and LSBMD analysis, overlap P-values for the knee OA and LSBMD comparison were at least one order of magnitude smaller. Four SNPs (rs7104420, rs9466056, rs881803 and rs4536164) overlapped at $P_t=5x10^{-4}$ in this analysis ($P_{perm} =4.21x10^{-3}$). Two of these, rs4536164 and rs9466056, fall within known BMD risk loci.[18]

Overlap signal was much weaker for the OA and FNBMD comparisons, with only five P_t reaching statistical significance (P_t = 0.001 for knee OA, P_t = 0.005 and 0.01 for hip OA, and P_t = 0.001 and P_t = 0.005 for combined OA). The SNP overlapping at $P_t=5x10^{-4}$ for hip OA and FNBMD (rs1524928) was also among the six SNPs identified in the hip OA and LSBMD analysis. Two SNPs overlapped at this P_t for knee OA, one being rs9466056 and the other rs1283614, which maps to an intron of the BMD locus *MEF2C*. [24]

Table 1 | SNP-based overlap analysis of OA and BMD.

	Combined OA				Hip OA				Knee OA			
	LSBMD		FNBMD		LSBMD		FNBMD		LSBMD		FNBMD	
Total number of SNPs	75,015		75,161		74,999		75,147		75,125		75,27	
Pt	SNPs	P_perm	SNPs	P_perm	SNPs	P_perm	SNPs	P_perm	SNPs	P_perm	SNPs	P_perm
0.5	28,940	8.53×10^{-2}	27,599	2.74×10^{-1}	28,563	1.64×10^{-1}	27,065	7.33×10^{-1}	28,910	7.95×10^{-1}	27,534	5.84×10^{-2}
0.1	2,633	7.92×10^{-2}	2,119	5.41×10^{-1}	2,513	1.46×10^{-2}	2,057	1.74×10^{-1}	2,620	3.67×10^{-2}	2,131	2.33×10^{-1}
0.05	931	1.25×10^{-2}	698	2.35×10^{-1}	868	1.42×10^{-2}	647	2.47×10^{-1}	939	8.98×10^{-4}	700	3.10×10^{-2}
0.04	682	2.41×10^{-3}	486	2.33×10^{-1}	641	6.48×10^{-4}	434	4.99×10^{-1}	680	4.74×10^{-4}	474	1.27×10^{-1}
0.03	451	1.20×10^{-3}	314	6.57×10^{-2}	416	1.02×10^{-3}	272	4.20×10^{-1}	437	2.87×10^{-3}	291	3.27×10^{-1}
0.02	251	1.95×10^{-3}	160	1.07×10^{-1}	239	6.10×10^{-5}	150	6.31×10^{-2}	244	1.59×10^{-3}	152	3.03×10^{-1}
0.01	87	2.79×10^{-2}	60	5.84×10^{-2}	87	2.97×10^{-4}	52	4.94×10^{-2}	88	3.01×10^{-3}	53	2.18×10^{-1}
0.005	37	6.89×10^{-3}	24	2.43×10^{-2}	38	3.04×10^{-4}	26	6.39×10^{-4}	29	1.97×10^{-1}	16	6.94×10^{-1}
0.001	10	3.90×10^{-5}	3	4.52×10^{-2}	7	1.68×10^{-3}	1	1	6	1.12×10^{-2}	4	3.39×10^{-2}
5×10^{-4}	4	6.81×10^{-3}	0	1	6	5.70×10^{-5}	1	5.64×10^{-2}	4	4.21×10^{-3}	2	7.12×10^{-2}

For each comparison, the total number of SNPs present in both datasets after LD-pruning is given, as well as the number of SNPs falling below each P-value threshold (Pt). P_{perm} = empirical overlap p-value obtained through permutation analysis

Evidence for Colocalising Regions

We employed a regional Bayesian colocalisation test that measures the posterior probabilities (PP) for each of four alternative hypotheses compared to one global null hypothesis (i.e. no associations in that region). We identified four independent genomic regions with a high posterior probability (PP) of harbouring one causal variant common to both traits analysed (PP for hypothesis 3 0.9) (**Table 2**). The region containing the *RPS6KA5* gene was identified by three comparisons (combined OA and LSBMD, hip OA and LSBMD, and hip OA and FNBMD). The most strongly associated SNPs in this region have genome-wide significant associations with increased BMD at both the LS and FN (rs1286147 and rs1286063, $P < 5x10^{-8}$) and nominally significant associations with increased risk of combined and hip OA (rs1286077, $P < 0.05$); the three SNPs are in perfect LD ($r^2=1.00$ for each pairwise combination).

Two further regions were identified in the hip OA and LSBMD analysis; one of these spans a known BMD locus close to the *KCNMA1* gene, while the other does not contain genome-wide significant variants for either OA or BMD.

The region identified in the knee OA and FNBMD analysis contains one lead SNP for both traits, rs9466056, which is a known variant associated with high FN and LSBMD mapping to an intergenic region between *CDKAL1* and *SOX4*.

We also identified three regions (**Table 2**) with a high PP of harbouring two distinct causal variants (PP for hypothesis 0.9). All three of these contain a known BMD locus, with the top SNPs for LSBMD mapping to introns of *IDUA, CCDC170* and *PDXDC1*. The top SNPs for knee and hip OA are nominally associated ($P < 0.05$) with these respective phenotypes in arcOGEN.

Gene and Pathway Analysis

Of the individual genes significantly associated ($Q < 0.05$) with at least one OA or BMD phenotype, *SUPTH3*, *COL11A1*, and *APCDD1* overlapped between OA and BMD (**Supplementary Material, Table S2**). All three include variants that were identified in the SNP-wise overlap analysis and taken forward for replication.

There were no pathways significantly associated with any OA phenotype in any of the analyses. One of the CP pathways was associated with FNBMD ("basal cell carcinoma", $Q = 0.02$) when allowing a 20 kilobase (kb) window around genes. Using GO annotations, a total of 33 unique pathways were associated with either BMD phenotype using strict or lenient gene definitions (**Supplementary Material, Tables S3-S5**), including several with direct biological relevance, such as "regulation of ossification" or "osteoblast development".

Table 2 | Regions with strong evidence of pleiotropy. For each region the number of SNPs, start and stop position in basepairs (bp) and most strongly associated SNPs for OA and BMD are given. Chromosome coordinates are in hg19. Hypothesis 3 = one causal variant; hypothesis 4 = two distinct causal variants; PP = posterior probability

	Analysis	SNPs	Chr	Start (bp)	Stop (bp)	Top SNP BMD	Top SNP OA	PP
Hypothesis 3	combinedOA and LSBMD	817	chr14	91297823	93129850	rs1286147; rs1286063	rs1286077	0.95
	hipOA and FNBMD	817	chr14	91297823	93129850	rs1286147	rs1286077	0.98
	hipOA and LSBMD	817	chr14	91297823	93129850	rs1286147; rs1286063	rs1286077	0.99
	hipOA and LSBMD	1242	chr10	78708452	80875213	rs7071206	rs716255	0.92
	hipOA and LSBMD	531	chr1	44974119	46897698	rs7554123	rs7545984	0.91
	kneeOA and FNBMD	1235	chr6	19208477	21677746	rs9466056	rs9466056	0.99
Hypothesis 4	hipOA and LSBMD	268	chr4	696848	1415698	rs3755955	rs3755920	0.97
	kneeOA and LSBMD	382	chr16	14464538	16152940	rs4985155	rs9935327	0.95
	kneeOA and LSBMD	1070	chr6	150255029	151910904	rs4869742	rs9384514	0.9

Cross-phenotype Meta-analysis

To search for potential novel associations not identified by single-trait GWAS, we performed a cross-phenotype meta-analysis between each pairwise combination of OA and BMD data-sets. Using the CPASSOC method,[25] we computed two statistics, S_{hom} and S_{het}, which assume homogeneous and heterogeneous effects across studies, respectively. The quantile– quantile and Manhattan plots of these analyses are shown in **Supplementary Material, Figures S1–S4**. We identified 13 independent associations not previously reported for BMD or OA, which we followed up in the UK Biobank combined OA dataset (**Supplementary Material, Table S10**). One SNP, rs11164649, was nominally significant (P < 0.05). This SNP lies in an intron of the COL11A1 gene and is in strong LD (r^2=0.92) with a variant (rs1903787) identified in the SNP-wise overlap analysis which was taken forward for replication.

Replication and Meta-analysis for OA

We meta-analysed 143 SNPs identified in the colocalisation and/or P-value based overlap analysis in arcOGEN, UK Biobank and deCODE (**Supplementary Material, Table S6**). None of the SNPs taken forward are genome-wide significantly associated with arcOGEN. We found a significant excess of independent SNPs with the same direction of effect in all three meta-analysis cohorts among variants with P_{meta}<0.05 (binomial sign test P = $7.75x10^{-11}$), as well as all independent SNPs included in the meta-analysis (binomial sign test P = 0.03).

Variants within several genes linked to bone, cartilage and extracellular matrix biology, including *APCDD1, SUPTH3, COL11A1, NOTCH4, SEMA3A, LGR4, PTCH1* and *RPS6KA5*, were associated at P_{meta}<0.05 (**Supplementary Material, Table S6**).

Two variants reached genome-wide significance in the meta-analysis across arcOGEN, deCODE and UK Biobank: rs12901071 (OR 1.08 95% CI 1.05-1.11, $P_{meta}=3.12x10^{-10}$) and rs10518707 (OR 1.07, 96% CI 1.03–1.09, $P_{meta}=2.15x10^{-8}$). Both are intronic variants in the *SMAD3* gene (r^2=0.645) and were identified in the SNP-wise overlap analysis of combined OA vs. LSBMD and hip OA vs. LSBMD, respectively.

Both new genome-wide significant SNPs for OA were imputed in the arcOGEN data (imputation info score > 0.95) and are nominally associated with combined OA (**Supplementary Material, Table S6; Figure S2**). They are also nominally associated with increased LSBMD in GEFOS (rs12901071, P = $1.58x10^{-3}$ and rs10518707, P = $3.47x10^{-5}$) (**Figure 2**), but not FNBMD (rs12901071, P = $3.72x10^{-1}$ and rs10518707, P = $2.46^{x}10^{-1}$). *SMAD3* is associated (P < 0.05) with LS and FNBMD, hip and combined OA in the gene analysis (**Supplementary Material, Table S7**), although this association only holds for LSBMD after using false discovery rate (FDR) correction (Q = $6.92x10^{-6}$).

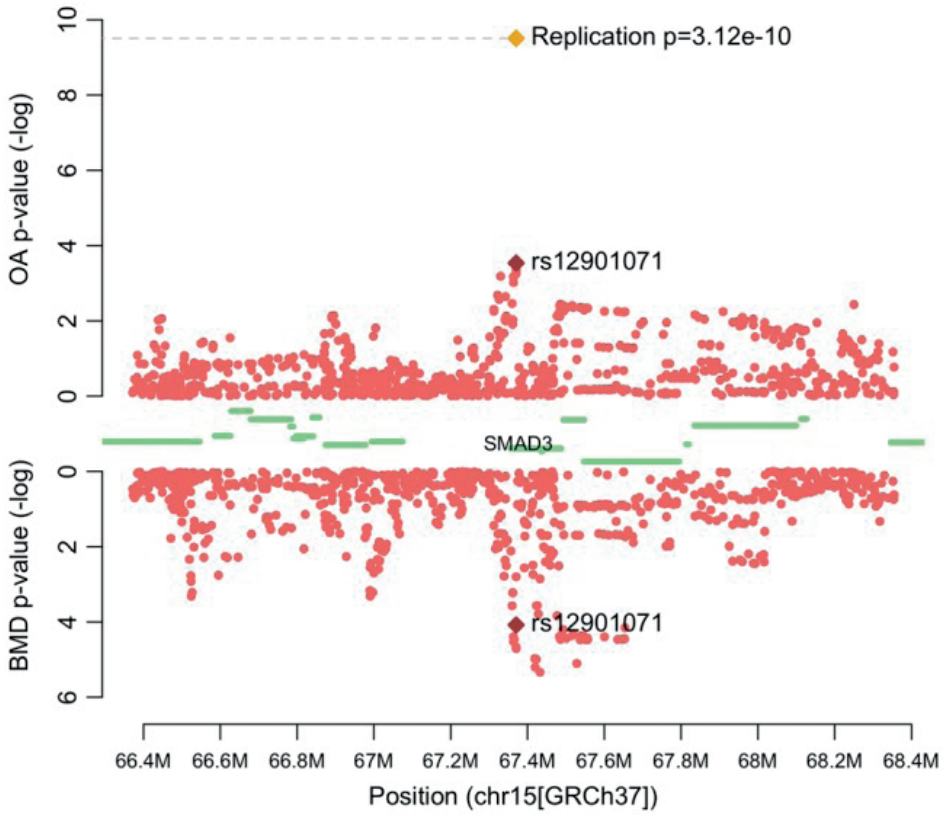


Figure 2 | Regional association plot of *SMAD3*. The -log(p-values) of SNPs in the arcOGEN combined osteoarthritis (OA) data (top) and GEFOS lumbar spine bone mineral density (LSBMD) data (bottom) are plotted against their chromosomal position. The meta-analysis p-value of rs12901071 is plotted as a golden diamond. Protein coding genes are represented by green bars

In the colocalisation analysis, the region in which both top SNPs reside (chr15:67,095,629-69,017,421) has a PP of containing a single pleiotropic variant associated with hip OA and LSBMD (hypothesis 3) of 0.88.

Functional Follow-up of SMAD3

Using RNA sequencing data, we confirmed the expression of *SMAD3* in low-grade degenerate articular cartilage of 12 knee and 9 hip OA patients undergoing total joint replacement [26] (**Supplementary Material, Figure S5**). SMAD3 is among the 30% most expressed genes in the knee articular cartilage samples, and among the 15% most expressed genes in the hip articular cartilage samples.

DISCUSSION

The analysis of shared genetic aetiology across epidemiologically linked traits can enhance power to identify disease variants and shed light into the biological mechanisms underpinning these associations. We conducted the first genome-wide overlap analysis of BMD and OA using summary statistics from the two largest GWAS of these two traits.

We identified 143 variants with evidence for association with OA and BMD. Many of these reside in or near biologically relevant genes, two of which (*KLHL42/KLHDC5* and *SUPT3H/ RUNX2*) are established loci for both traits.[6,18] Variants in three loci (*SUPTH3, APCDD1*, and *COL11A1*) were also significantly associated with at least one OA and BMD phenotype in the gene analysis. *APCDD1* is an inhibitor of WNT signaling,[27] which is implicated in both OA and BMD. *COL11A1* encodes collagen type 11, an important component of cartilage and bone, and has been associated with OA in a candidate gene meta-analysis .[28] Other examples include the *LGR4* gene, in which a rare variant in the Icelandic population has been associated with low BMD and osteoporotic fractures;[29] and *SEMA3A*, which affects bone remodeling in rats.[30]

We identified novel genome-wide significant associations at two intronic SNPs in *SMAD3*, and confirm expression of this gene in primary chondrocytes from articular cartilage of OA patients undergoing total joint replacement surgery. Activated SMAD3 acts downstream of TGF-b, repressing osteoblast differentiation and the production of bone matrix .[31,23] It also represses the cartilage-degrading enzyme matrix metalloproteinase 13 in chondrocytes. [32] Missense mutations in a conserved protein domain of *SMAD3* have been linked to aneurysm-osteoarthritis syndrome, a congenital disorder characterised by arterial aneurysms, heart abnormalities and early-onset OA.[33]

Due to its role in bone and cartilage biology, *SMAD3* has been previously assessed in a candidate gene study of hip and knee OA.[34] Despite their small sample size (number of cases < 400), the investigators found nominal associations (P < 0.05) for both OA phenotypes in their discovery, which were further strengthened in a meta-analysis (hip OA P = $4x10^{-4}$; knee OA P = $7.5x10^{-6}$). Notably, their top signal (rs12901499) maps to the same locus as our lead SNP (r^2=0.645).

More recently, two studies have shown *SMAD3* expression to be correlated with the genotype at a 3'UTR SNP, [35] and to be significantly higher in cartilage from OA patients compared to healthy controls.[36] The authors postulate that this could be a compensatory mechanism to counteract existing cartilage damage, or that *SMAD3* expression levels outside a narrow range have detrimental effects.

We observed a stronger overlap between LSBMD and OA. The fact that only the correlation between combined OA and LSBMD was significant could be due to the bigger sample size in this OA dataset compared to the hip or knee OA data. While

the FN- and LSBMD datasets are very similar in size, the knee OA and hip OA datasets each contain approximately half the number of cases compared to the combined OA dataset. This difference in power might at least partly explain why the genetic correlation estimates for joint-specific OA and LSBMD did not achieve statistical significance. Epidemiological data from the Chingford study have shown increased baseline BMD to be associated with incident radiographic knee OA, with the mean in-crease in LSBMD being approximately twice as high as the increase in FNBMD.[10,15] Incident knee OA was also linked to higher baseline LSBMD, but not FNBMD, in the Baltimore Longitudinal Study of Ageing.[37] The reasons for this differential association of FN- and LSBMD with OA remain unclear. One possible explanation could be the comorbidity of knee and spinal OA, characterised by spinal osteophytes, which could lead to increased LSBMD measurements. However, in one study, adjustment for the presence of osteophytes at the lumbar spine did not change the strength of association between OA and LSBMD.[10] Damage to the spine accumulates over time and can lead to changes such as breakdown of the vertebral discs, scoliosis and osteochondrosis. This process is also referred to as degenerative disc disease (DDD). Although the association between DDD and LSBMD remains inconclusive,[38-41] it is known that the presence of degenerative features can increase LSBMD measurements obtained via dual X-ray absorptiometry.[42] While this may have contributed to the observed association between LSBMD and OA, we found genetic correlations of a similar magnitude between OA and skull, as well as total body BMD measurements in a pediatric cohort [43](Supplementary Material, Table S11; Supplementary Methods). As DDD and related features such as osteophytes are unlikely to be present in young individuals, these results suggest that the correlation between OA and LSBMD is not purely artefactual.

Our analyses showed a greater degree of overlap between hip OA and both BMD measurements than between knee OA and BMD. Hip OA is estimated to have a higher heritability than knee OA,[44] with environmental risk factors such as physical activity and BMI more strongly associated with the latter.[45]

The analyses outlined here present the first comprehensive evaluation of genetic overlap between BMD and radiographic OA. Our results lend further support to the hypothesis of common genetic factors underlying these two traits, and establish *SMAD3* as a genome-wide significant risk locus for OA with a potential pleiotropic effect on BMD.

Our work exemplifies the potential to uncover new disease risk loci by combining data of epidemiologically linked traits. Recent efforts in the development of statistical methods to detect pleiotropy, as well as the establishment of cross-disorder working groups[46] and of biobank collections,[47] have made it possible to systematically assess pleiotropy in human disease genetics. Methods combining univariate summary statistics of different traits –s uch as the colocalisation analysis employed here [48] - often

do not require a locus to be genome-wide significantly associated with any of the individual studies to detect a cross-phenotype association. Hence, they can increase power to identify associated variants or regions without the need to collect larger sample sizes.[49]

There is a stark difference in sample size and, consequently, statistical power between the arcOGEN and GEFOS GWAS data-sets. Larger datasets where phenotype information for both OA and BMD is available in the same individuals will be necessary to further disentangle the extent of shared genetics between them. Pinpointing the common biological pathways of these two complex traits will provide insight into the underlying mechanisms of OA, facilitating the identification of novel targets or drug repurposing opportunities for its treatment.

MATERIALS AND METHODS

Datasets

All analyses outlined here were conducted using summary association statistics from the arcOGEN [6] and GEFOS consortia. [18] The arcOGEN data comprised three OA phenotypes: knee OA, hip OA, and knee and/or hip OA (combined OA). A detailed description of the contributing studies and phenotype definitions can be found in.[18] Briefly, OA case status was determined radiographically as a Kellgren-Lawrence grade score 2. Most cases included in arcOGEN had progressed to a severe disease endpoint, as evident from the fact that 80% had undergone total joint replacement surgery.

For the P-value-based overlap analysis as well as the gene and pathway analysis, we excluded samples from London-based cohorts (TwinsUK and Chingford Study) from the arcOGEN data-sets to avoid overlap with GEFOS. After exclusion of 714 samples, we carried out genome-wide association analyses on the arcOGEN dataset for each of the three phenotype groups using the "–method score" option in SNPTEST v2.5.[50] The full arcOGEN dataset was used for all other analyses described.

The BMD data consisted of meta-analysis summary statistics for FN and LSBMD. [48]

For replication, we used summary statistics from two OA GWAS: the UK Biobank [47] and the deCODE study. Details on case definitions in UK Biobank and deCODE can be found in the Supplementary Methods. Sample sizes for each dataset are outlined in **Supplementary Material, Table S8**.

Genome-wide Correlation

We performed LD score regression analysis [51] on each pairwise combination between the BMD and OA datasets, using pre-computed LD scores based on the European sample of the 1000 Genomes Project.[52] We accounted for sample overlap between

each pair of datasets through tetrachoric correlation of the Z-scores of SNPs present in both studies.

Assessment of Shared Association Signals

For each pairwise combination between the two BMD and three OA phenotypes we assessed the extent of shared signals at different p-value cutoffs, following the approach described by Elliott and colleagues.[53] Briefly, we filtered both datasets to a common set of SNPs on which p-value-informed LD pruning was performed. To this end, SNPs were sorted based on their association with OA, and, starting with the lowest p-value, any SNP in LD with the index SNP (r2>0.05) was removed. The next SNP was then considered, and so on.

The extent of shared association signals between OA and BMD was assessed by counting the number of overlapping variants above and below ten different p-value thresholds (Pt: 0.5, 0.1, 0.05, 0.04, 0.03, 0.02, 0.01, 0.005, 0.001, 5×10^{-5}). To test for significance of overlap, a chi-squared test on the resulting 2×2 contingency tables was performed at each Pt.

Empirical overlap p-values were obtained by repeating the chi-squared test after randomly permuting the GEFOS p-values. This was done 1,000,000 times to obtain a null distribution of overlap p-values against which the original overlap p-value could be compared.

Colocalisation Analysis

We employed a Bayesian colocalisation method to search for genomic regions harbouring cross-phenotype associations be-tween OA and BMD. [48] Briefly, the algorithm uses Z-scores and standard errors from two association studies to generate posterior probabilities for each of five hypotheses:

H_0: the region contains no variants associated with trait 1 or trait
H_1: the region contains one variant associated with trait 1
H_2: the region contains one variant associated with trait 2
H_3: the region contains one variant associated with both trait 1 and trait 2
H_4: the region contains one variant associated with trait 1 and a second variant associated with trait 2

Genomic regions were defined according to approximately independent LD-blocks (54). Analogous to LD score regression, we used the tetrachoric correlation of the Z-scores of SNPs pre-sent in datasets as an estimate of sample overlap.

Gene and Pathway Analysis

Gene- and pathway analyses were performed on each OA and BMD dataset using MAGMA.[55] First, SNPs are assigned to genes, which are tested for their association with the phenotype. Results from this step are then combined into pathway-based association statistics. For the gene analysis, we grouped variants into genes using SNP locations from dbSNP version 135 and NCBI 37.3 gene definitions. We performed this step twice, once annotating SNPs to a gene only if they fell within the gene's transcription start and stop site, and once including SNPs that fell within a 20 kilobase window of the gene. We ran two separate pathway analyses, one using the Molecular Signatures Database canonical pathways collection,[56] comprising 1,329 manually curated gene-sets from nine databases (Supplementary Material, Table S9), and one using 6,166 gene-sets from the Gene Ontology pathway database.[57] Significance was defined using a 5% FDR equivalent to a Q-value of 0.05 for both the gene and pathway analyses. [58]

Cross-phenotype Meta-analysis

We used a multi-trait extension to meta-analysis to search for novel associations in each pairwise combination of arcOGEN and GEFOS datasets. [25] The method, CPASSOC, requires only summary statistics and generates two test statistics: the first, S_{hom}, assumes homogeneous effects across studies and is equivalent to performing an inverse variance weighted meta-analysis if no sample overlap between the studies exists. The second, S_{het}, is more powerful if effects are heterogeneous between studies. Both statistics require the specification of a correlation matrix between the included studies. To calculate this, we followed the approach by Park et al.,[59] using all independent SNPs ($r^2 < 0.2$) present in both the OA and BMD dataset that were not associated with either trait ($1.96 > Z$ score < 1.96), and taking the Pearson's correlation of their Z-scores.[25] We performed an in-silico lookup in the UK Biobank hip and/ or knee OA data of top SNPs with $P < 5\ 10^{-8}$ in any of the CPASSOC analyses that did not fall into known OA or BMD loci.

Replication and Meta-analysis for OA

We took forward a total of 143 SNPs for in silico replication. This set comprises the two most strongly associated variants (one for each trait) in each region from the Bayesian colocalisation test, as well as all variants overlapping at P_t=0.005 in the SNP-based overlap analyses. We used the METAL [60] software package to perform inverse variance weighted meta-analysis of these SNPs in relation to hip and/or knee OA using summary statistics from the arcOGEN combined OA dataset (including London samples), the UK Biobank [47] and the deCODE [61] study.

Functional Follow-up of SMAD3

Samples were obtained and processed as in Ref. [25] Briefly, articular cartilage was obtained from 12 patients undergoing total joint replacement for knee OA, and 9 patients for hip OA. Cartilage was graded using the OARSI cartilage classification system.[62,63]

Detailed acknowledgments and online resources can be found in the published article online

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REFERENCES

1. Zhang Y., Jordan J.M. (2010) Epidemiology of osteoarthritis. Clin. Geriatr. Med., 26, 355–369.
2. Glyn-Jones S., Palmer A.J.R., Agricola R., Price A.J., Vincent T.L., Weinans H., Carr A.J. (2015)Osteoarthritis.Lancet,386, 376–387.
3. Hochberg M.C., Yerges-Armstrong L., Yau M., Mitchell B.D. (2013) Genetic epidemiology of osteoarthritis: recent developments and future directions. Curr. Opin. Rheumatol., 25, 192–197.
4. Zengini E., Finan C., Wilkinson J.M. (2016)The genetic epidemiological landscape of hip and knee osteoarthritis: Where are we now and where are we going? J. Rheumatol., 43, 260–266.
5. Panoutsopoulou K., Southam L., Elliott K.S., Wrayner N., Zhai G., Beazley C., Thorleifsson G., Arden N.K., Carr A., Chapman K. et al. (2011)Insights into the genetic architecture of osteoarthritis from stage 1 of the arcOGEN study.Ann. Rheum. Dis.,70, 864–867. 6.Zeggini E., Panoutsopoulou K., Southam L., Rayner N.W., Day-Williams A.G., Lopes M.C., Boraska V., Esko T., Evangelou E., Hofman A. et al. (2012)Identification of new susceptibility loci for osteoarthritis (arcOGEN): A genome-wide association study. Lancet, 380, 815–823. 7.Sivakumaran S., Agakov F., Theodoratou E., Prendergast J.G., Zgaga L., Manolio T., Rudan I., McKeigue P., Wilson J.F., Campbell H. (2011) Abundant pleiotropy in human complex diseases and traits.Am. J. Hum. Genet.,89, 607–618
8. Foss M.V., Byers P.D. (1972) Bone density, osteoarthrosis of the hip, and fracture of the upper end of the femur.Ann. Rheum. Dis.,31, 259–264. 9.Nevitt M.C., Zhang Y., Javaid M.K., Neogi T., Curtis J.R., Niu J., McCulloch C.E., Segal N.A., Felson D.T. (2010) High systemic bone mineral density increases the risk of incident knee OA and joint space narrowing, but not radiographic progression of existing knee OA: the MOST study. Ann. Rheum. Dis.,69, 163–168.
10. Hart D.J., Mootoosamy I., Doyle D.V., Spector T.D. (1994)The relationship between osteoarthritis and osteoporosis in the general population: the Chingford Study. Ann. Rheum. Dis.,53, 158–162.
11. Hannan M.T., Anderson J.J., Zhang Y., Levy D., Felson D.T. (1993)Bone mineral density and knee osteoarthritis in elderly men and women. the framingham study.Arthritis Rheum.,36, 1671–1680. 12.Hardcastle S. a., Dieppe P., Gregson C.L., Smith G.D., Tobias J.H. (2015)Osteoarthritis and bone mineral density: are strong bones bad for joints? Bonekey Rep., 4, 1–8. 13.Hardcastle S. a., Dieppe P., Gregson C.L., Arden N.K., Spector T.D., Hart D.J., Edwards M.H., Dennison E.M., Cooper C., Sayers A. et al. (2015) Individuals with high bone mass have an increased prevalence of radiographic knee osteoarthritis. Bone, 71, 171–179. 14.Bettica P., Cline G., Hart D.J., Meyer J., Spector T.D. (2002)Evidence for increased bone resorption in patients with progressive knee osteoarthritis: Longitudinal results from the Chingford study.Arthritis Rheum.,46, 3178–3184.
15. Hart D.J., Cronin C., Daniels M. (2002) The relationship of bone density and fracture to incedent and progressive radiographic osteoarthritis of the knee. Arthritis Rheum., 46, 92–99.
16. Zhang Y., Hannan M.T., Chaisson C.E., McAlindon T.E., Evans S.R., Aliabadi P., Levy D., Felson D.T. (2000) Bone mineral density and risk of incident and progressive radiographic knee osteoarthritis in women: the Framingham Study.J. Rheumatol.,27, 1032–1037.

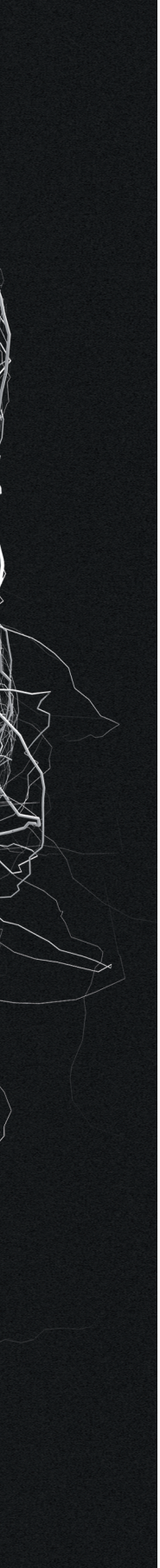
17. Bergink A.P., Uitterlinden A.G., Van Leeuwen J.P.T.M., Hofman A., Verhaar J.A.N., Pols H.A.P. (2005) Bone mineral density and vertebral fracture history are associated with incident and progressive radiographic knee osteoarthritis in elderly men and women: the Rotterdam Study.Bone,37, 446–456.
18. Estrada K., Styrkarsdottir U., Evangelou E., Hsu Y.H., Duncan E.L., Ntzani E.E., Oei L., Albagha O.M.E., Amin N., Kemp J.P. et al. (2012) Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat. Genet., 44, 494–501.
19. Zheng H., Forgetta V., Hsu Y., Estrada K., Rosello-Diez A., Leo P.J., Dahia C.L., Park-Min K.H., Tobias J.H., Kooperberg C. et al. (2015) Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture.Nature,526, 112–117.
20. Geusens P.P., van den Bergh J.P. (2016) Osteoporosis and osteoarthritis: shared mechanisms and epidemiology.Curr. Opin. Rheumatol.,28, 97–103.
21. Balooch G., Balooch M., Nalla R.K., Schilling S., Filvaroff E.H., Marshall G.W., Marshall S.J., Ritchie R.O., Derynck R., Alliston T. (2005) TGF-beta regulates the mechanical properties and composition of bone matrix.Proc. Natl. Acad. Sci. U. S. A,102, 18813–18818. 22.Panoutsopoulou K., Zeggini E. (2013) Advances in osteoarthritis genetics. J. Med. Genet., 50, 715–724.
23. Yerges-Armstrong L.M., Yau M.S., Liu Y., Krishnan S., Renner J.B., Eaton C.B., Kwoh C.K., Nevitt M.C., Duggan D.J., Mitchell B.D. et al. (2014)Association analysis of BMD-associated SNPs with knee osteoarthritis.J. Bone Miner. Res.,29, 1373–137924.Rivadeneira F., Styrkársdottir U., Estrada K., Halldórsson B.V., Hsu Y.-H., Richards J.B., Zillikens M.C., Kavvoura F.K., Amin N., Aulchenko Y.S. et al. (2009)Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies.Nat. Genet.,41, 1199–1206.
25. Zhu X., Feng T., Tayo B.O., Liang J., Young J.H., Franceschini N., Smith J. a., Yanek L.R., Sun Y.V., Edwards T.L. et al. (2015) Meta-analysis of correlated traits via summary statistics from GWASs with an application in hypertension.*Am.*J. Hum. Genet.,96, 21–36.
26. Steinberg J., Ritchie G.R.S., Roumeliotis T.I., Jayasuriya R.L., Brooks R.A., Binch A.L.A., Shah K.M., Coyle R., Pardo M., Le Maitre C.L. et al. (2016) Integrative epigenomics, transcriptomics and proteomics of patient chondrocytes reveal genes and pathways involved in osteoarthritis.*bioRxiv*
27. Shimomura Y., Agalliu D., Vonica A., Luria V., Wajid M., Baumer A., Belli S., Petukhova L., Schinzel A., Brivanlou A.H. et al. (2010)APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex.Nature,464, 1043–1047.
28. Rodriguez-Fontenla C., Calaza M., Evangelou E., Valdes A.M., Arden N., Blanco F.J., Carr A., Chapman K., Deloukas P., Doherty M. et al. (2014)Assessment of osteoarthritis candidate genes in a meta-analysis of nine genome-wide association studies. Arthritis Rheumatol.,66, 940–949.
29. Styrkarsdottir U., Thorleifsson G., Sulem P., Gudbjartsson D.F., Sigurdsson A., Jonasdottir A., Jonasdottir A., Oddsson A., Helgason A., Magnusson O.T. et al. (2013)Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits.Nature,497, 517–520. 30.Fukuda T., Takeda S., Xu R., Ochi H., Sunamura S., Sato T., Shibata S., Yoshida Y., Gu Z., Kimura A. et al. (2013) Sema3A regulates bone-mass accrual through sensory innervations.Nature,497, 490–493.
31. Alliston T., Choy L., Ducy P., Karsenty G., Derynck R., Akiyoshi S., Inoue H., Hanai J., Kusanagi K., Nemoto N. et al. (2001) TGF-beta-induced repression of CBFA1 by Smad3 decreases

cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. embo J., 20, 2254–2272.
32. Chen C.G., Thuillier D., Chin E.N., Alliston T. (2012)Chondrocyte-intrinsic Smad3 represses Runx2-inducible matrix metalloproteinase 13 expression to maintain articular cartilage and prevent osteoarthritis.Arthritis Rheum.,64, 3278–3289.
33. van de Laar I.M.B.H., Oldenburg R.A., Pals G., Roos-Hesselink J.W., de Graaf B.M., Verhagen J.M.A., Hoedemaekers Y.M., Willemsen R., Severijnen L.-A., Venselaar H. et al. (2011)Mutations in SMAD3 cause a syndromic form of aortic aneurysms and dissections with early-onset osteoarthritis.Nat. Genet.,43, 121–126.
34. Valdes A.M., Spector T.D., Tamm A., Kisand K., Doherty S.A., Dennison E.M., Mangino M., Tamm A., Kerna I., Hart D.J. et al. (2010)Genetic variation in the SMAD3 gene is associated with hip and knee osteoarthritis.Arthritis Rheum.,62, 2347–2352.
35. Raine E.V.A., Reynard L.N., van de Laar I.M.B.H., Bertoli-Avella A.M., Loughlin J. (2014)Identification and analysis of a SMAD3 cis-acting eQTL operating in primary osteoarthritis and in the aneurysms and osteoarthritis syndrome.Osteoarthr. Cartil.,22, 698–705. 36.Aref-Eshghi E., Liu M., Razavi-Lopez S.B., Hirasawa K., Harper P.E., Martin G., Furey A., Green R., Sun G., Rahman P. et al. (2016)SMAD3 is upregulated in human osteoarthritic cartilage independent of the promoter DNA methylation.J. Rheumatol.,43, 388–394.
37. Hochberg M.C., Lethbridge-Cejku M., Tobin J.D. (2004)Bone mineral density and osteoarthritis: Data from the Baltimore Longitudinal Study of Aging.Osteoarthr. Cartil.,12, 45–48.
38. Harada A., Okuizumi H., Miyagi N., Genda E. (1998) Correlation between bone mineral density and intervertebral disc degeneration.Spine (Phila. Pa. 1976),23, 857–861.
39. Grams A.E., Rehwald R., Bartsch A., Honold S., Freyschlag C.F., Knoflach M., Gizewski E.R., Glodny B. (2016) Correlation between degenerative spine disease and bone marrow density: a retrospective investigation.BMC Med. Imaging,16, 17.
40. Pye S.R., Reid D.M., Adams J.E., Silman A.J., O'Neill T.W. (2006)Radiographic features of lumbar disc degeneration and bone mineral density in men and women. Ann. Rheum. Dis.,65, 234–238.
41. Miyakoshi N., Itoi E., Murai H., Wakabayashi I., Ito H., Minato T. (2003) Inverse relation between osteoporosis and spondylosis in postmenopausal women as evaluated by bone mineral density and semiquantitative scoring of spinal degeneration. Spine (Phila. Pa. 1976),28, 492–495.
42. Rand T., Seidl G., Kainberger F., Resch A., Hittmair K., Schneider B., Glüer C.C., Imhof H. (1997)Impact of spinal degenerative changes on the evaluation of bone mineral density with dual energy X-ray absorptiometry (DXA).Calcif. Tissue Int.,60, 430–433.
43. Kemp J.P., Medina-Gomez C., Estrada K., St Pourcain B., Heppe D.H.M., Warrington N.M., Oei L., Ring S.M., Kruithof C.J., Timpson N.J. et al. (2014)Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment.PLoS Genet.,10, e1004423.
44. Valdes A.M., Spector T.D. (2011)Genetic epidemiology of hip and knee osteoarthritis.Nat. Rev. Rheumatol.,7, 23–32.
45. Blagojevic M., Jinks C., Jeffery A., Jordan K.P. (2010)Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis.Osteoarthr. Cartil.,18, 24–33.
46. Smoller J.W. (2013)Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis.Lancet,381, 1371–1379.

47. Sudlow C., Gallacher J., Allen N., Beral V., Burton P., Danesh J., Downey P., Elliott P., Green J., Landray M. et al. (2015)UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age.PLoS Med.,12, e1001779.
48. Pickrell J., Berisa T., Segurel L., Tung J.Y., Hinds D. (2015) Detection and interpretation of shared genetic influences on 42 human traits.*Nat. Genet.*,**48,**709–717.
49. Solovieff N., Cotsapas C., Lee P.H., Purcell S.M., Smoller J.W. (2013)Pleiotropy in complex traits: challenges and strategies.Nat. Rev. Genet.,14, 483–495.
50. Marchini J., Howie B., Myers S., McVean G., Donnelly P. (2007)A new multipoint method for genome-wide association studies by imputation of genotypes.Nat. Genet.,39, 906–913.
51. Bulik-Sullivan B., Finucane H.K., Anttila V., Gusev A., Day F.R., Loh P.-R. ReproGen Consortium, Psychiatric Genomics Consortium, Genetic Consortium for Anorexia Nervosa of the Wellcome Trust Case Control Consortium 3 Duncan L. et al. (2015) An atlas of genetic correlations across human diseases and traits.Nat. Genet.,47, 1236–1241.
52. Bulik-Sullivan B.K., Loh P.-R., Finucane H.K., Ripke S., Yang J. Schizophrenia Working Group of the Psychiatric Genomics Consortium Patterson N., Daly M.J., Price A.L., Neale B.M. (2015)LD Score regression distinguishes confounding from polygenicity in genome-wide association studies.Nat. Genet., advance on, 291–295.
53. Elliott K.S., Chapman K., Day-Williams A., Panoutsopoulou K., Southam L., Lindgren C.M., Arden N., Aslam N., Birrell F., Carluke I. et al. (2013) Evaluation of the genetic overlap between osteoarthritis with body mass index and height using genome-wide association scan data.Ann. Rheum. Dis.,72, 935–941.
54. Berisa T., Pickrell J.K. (2016)Approximately independent linkage disequilibrium blocks in human populations.Bioinformatics,32, 283–285.
55. de Leeuw C.A., Mooij J.M., Heskes T., Posthuma D. (2015) MAGMA: generalized gene-set analysis of GWAS data.PLoS Comput. Biol.,11, e1004219.
56. Subramanian A., Tamayo P., Mootha V.K., Mukherjee S., Ebert B.L., Gillette M.A., Paulovich A., Pomeroy S.L., Golub T.R., Lander E.S. et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.Proc. Natl. Acad. Sci.,102, 15545–15550.
57. Ashburner M., Ball C.A., Blake J.A., Botstein D., Butler H., Cherry J.M., Davis A.P., Dolinski K., Dwight S.S., Eppig J.T. et al. (2000)Gene ontology: tool for the unification of biology.Nat. Genet.,25, 25–29.
58. Benjamini Y., Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing.J. R. Stat. Soc. Ser. B,57, 289–300.
59. Park H., Li X., Song Y.E., He K.Y., Zhu X. (2016) Multivariate analysis of anthropometric traits using summary statistics of genome-wide association studies from GIANT Consortium.PLoS One,11, e0163912.
60. Willer C.J., Li Y., Abecasis G.R. (2010)METAL: Fast and efficient meta-analysis of genome-wide association scans.Bioinformatics,26, 2190–2191.
61. Gudbjartsson D.F., Helgason H., Gudjonsson S.A., Zink F., Oddson A., Gylfason A., Besenbacher S., Magnusson G., Halldorsson B.V., Hjartarson E. et al. (2015) Large-scale whole-genome sequencing of the Icelandic population.Nat. Genet.,47, 435–444.
62. Mankin H.J., Dorfman H., Lippiello L., Zarins A. (1971) Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips: II. correlation of morphology with biochemical and metabolic data.J. Bone Jt. Surg.,53, 523–537.

63. Pearson R.G., Kurien T., Shu K.S.S., Scammell B.E. (2011)Histopathology grading systems for characterisation of human knee osteoarthritis – reproducibility, variability, reliability, correlation, and validity.Osteoarthr. Cartil.,19, 324–331.





5

General Discussion

EPIDEMIOLOGY, PREVENTION AND MANAGEMENT OF OSTEOPOROTIC FRACTURES

In Europe, 3 million fractures occur each year accounting for 2 million disability-adjusted life years.[1] The fracture incidence varies between and within countries, which can be attributed to diverse factors including age, sex, geography, ethnicity, and socioeconomic status. [2,3,4,5] In the Rotterdam Study the annual incidence of non-vertebral fracture between 1989 and 2013 was 21 cases per thousand persons from which the majority experienced hip, wrist or humerus fracture. [2] In addition, we observed a plateau in the incidence of all-type and site-specific non-vertebral fractures between 1989-2001 and 2001-2013. [2] Worldwide, cohort studies have reported similar trends, with some even showing decrease in fracture incidence. [6,7,8,9,10] Even though fragility fracture incidence rates might remain stable, demographic changes in the decades to follow will lead to an increase in the absolute number of fracture cases. It has been projected that by the end of 2050 the number of hip fractures worldwide will range between 7.3 and 21.3 millions. [11] Nevertheless, I believe that with our current understanding of the epidemiology, pathophysiology, and management of fragility fractures, we can significantly reduce these numbers. Thus, identifying people at increased fracture risk and implementing preventive measures and subsequent targeted interventions should be of paramount importance for decreasing the number and burden of fragility fractures in the years to come.

Identifying People at High Fracture Risk

Fractures in elderly people are typically consequence of osteoporosis. The cornerstone of diagnosis and management of osteoporosis is BMD. Likewise, low BMD is a strong predictor of future fractures in postmenopausal women and elderly men. [12] Some of my work showed that one measurement of femoral neck BMD after the age of 45 years could predict fractures over 20-23 years in both men and women (**Chapter 2.1.**). As anti-osteoporotic treatment reduces future fracture risk for individuals with osteoporosis, the BMD assessment can identify people at risk who might benefit from treatment. However, BMD has high specificity but low sensitivity in predicting fractures as most fractures still occur in individuals with BMD above the osteoporosis treatment threshold (**Chapter 2.1.1**). Therefore, measuring BMD might offer more benefits in diagnosis and treatment than for fracture risk stratification. The current therapeutic approach focuses on using a clinical threshold, established as a T-score lower than 2.5; but in practice a T-score of -2.5 and a T-score of -2.2 do not represent much risk difference. Further, treatment is only indicated in the former case leaving the latter group at imminent risk of fracture without intervention. Thus, intervention thresholds for identifying a fraction of individuals with considerable fracture risk

should not be the function of a fixed T-score. [13] Nevertheless, by considering simultaneously clinical risk factors for osteoporosis (such as age, family history, together with secondary causes of osteoporosis) in prediction models, we can increase the sensitivity of BMD. These clinical factors have been successfully incorporated in different algorithms and risk assessment tools such as the Garvan, [14] QFracture, [15] and FRAX [16] which have been shown to improve treatment decision-making. For instance, an individual with osteopenia lacks treatment indication by the BMD-value alone; yet, treatment will be indicated for the same individual if on top of the BMD measurement, a history of fragility fracture or a very high risk based on the FRAX algorithm is present. [16] Nevertheless, there is room to improve further the existing fracture risk algorithms. One immense pitfall of the FRAX algorithm is the omission of falls history in the prediction model, even though its inclusion has been shown to enhance the predictive capacity of the model. [17] Further, current FRAX efforts are attempting to include muscle mass and function, as they are likely causally-related with fracture risk; [18] yet, muscle strength and function are also fall-related risk factors. [19]

I also investigated whether sarcopenia augments the risk of fracture in osteoporotic individuals, given that sarcopenia is considered analogous to osteoporosis, but then for muscle- instead of bone-mass loss. Indeed, some of my work showed that pre-sarcopenic and sarcopenic individuals have higher prevalence of non-vertebral fractures compared to the rest of our study population. However, these differences were notoriously driven by age and sex (**Chapter 3.2**). Moreover, the strength of the association between osteoporosis and prevalent fractures remained essentially unchanged in the presence of sarcopenia (**Chapter 3.2**). Current evidence shows that DXA- or bioimpedance-derived lean mass is of very limited value in the prediction of incident fractures independent of BMD. [20] In contrast, alternative methods for assessing muscle mass, such as peripheral quantitative computed tomography (pQCT) or labelled creatinine dilution (D3-Creat) could provide valuable and/or more accurate information regarding muscle health and its effects on fracture. As such pQCT measurements allow the assessment of fat infiltration in muscle, while D3-Creat constitutes an unbiased determination of muscle mass independent of total water content. Yet, including muscle mass in the FRAX algorithm is unlikely to provide added value, whereas omitting falls history may be detrimental to its prognostic value. It is worthwhile mentioning that instead of muscle mass, measures of muscle function, such as physical performance, may have an independent effect on fractures [21] that should be explored extensively in future efforts.

Beyond BMD: Measures of Bone Geometry and Microstructure

In older adults, a large fraction of fractures can occur in the presence of osteopenic and even normal BMD levels [2] indicating that either the threshold needs to be optimized,

or that other bone parameters besides BMD may have an important contribution to fracture risk. Bone strength, which is the main determinant of fracture risk, is characterized by a variety of bone properties such as bone geometry, cortical thickness and porosity, trabecular bone morphology, and tissue mineralization density. [22] Under some assumptions, selected aspects of bone geometry and strength can be assessed on DXA scans with the use of hip strength analysis (HSA), like estimates of cortical thickness, cross-sectional area (CSA), cross-sectional moment of inertia (CSMI), section modulus (Z) and buckling ratio (BR). [23] The HSA approach is not free of limitations, assessing 3D aspects from a 2D projection. In contrast, 3D assessments of volumetric BMD and bone micro-architecture have recently gained momentum. Several non-invasive low-radiation methods such as pQCT can estimate some aspects of bone micro-architecture. The pQCT method provides volumetric BMD and can distinguish between cortical and trabecular BMD, which may exert distinct impact on the risk of fracture. [24] High-resolution p-QCT (HR-pQCT) can also measure parameters of micro-architecture (i.e., cortical porosity and trabecular number and connectivity), but these additional parameters remain highly correlated with trabecular and cortical BMD. In a set of approximately 560 individuals, my work showed that DXA-derived parameters of BMD (total body, femoral neck, and lumbar spine BMD) are moderately correlated with pQCT bone parameters at the tibia (trabecular density, cortical density and area) (**Chapter 3.1**). Interestingly, the DXA-derived areal BMD as compared to the pQCT cortical and trabecular BMD showed stronger correlation with strength stress index (SSI) (**Chapter 3.1**); an important pQCT-based indicator of fracture risk. This, further underscores the strength of DXA-derived BMD as a key fracture determinant. Trabecular microarchitecture can also be assessed from DXA images through the trabecular bone score (TBS). There is a growing body of evidence supporting the additive value of TBS in fracture risk prediction [25,26] i.e., TBS may hold additional clinical value, as a recent large scale meta-analysis reported that the TBS effect on major osteoporotic fractures is largely independent of BMD.[27] While initially TBS was only measured at the lumbar spine, recent efforts have allowed expanding its assessment to other skeletal sites and X-ray based technologies.

Fragility Fractures and Osteoporosis Care Gap

In Europe, the treatment gap of osteoporosis, i.e. number of people who need to be treated but are not, ranges between 25-95%; [28] being the lowest in Spain and highest in Bulgaria. As there are two sides to every story, there are two major problems for the substantial treatment gap in the osteoporosis field i.e., ***poor treatment initiation and poor treatment adherence.*** For start, despite the guidelines and available treatment options in clinical practice most patients suffering a fragility fracture do not receive medications to reduce the risk of future fracture in the year following the

fracture, nor are they evaluated for osteoporosis. For example, in the US Medicare population, up to 72% of women within 12 months of index fracture were untreated. [29] Similarly, in a Canadian study, only 20% of patients who sustained a traumatic fracture (predominantly of the distal radius, proximal humerus, or proximal femur) were evaluated for osteoporosis and received adequate osteoporotic treatment at 1-year follow-up. [30] Most importantly, this therapeutic gap has been shown to be particularly wide in the elderly people in whom the importance and impact of treatment is higher. [31] On the other hand, a significant proportion of the treated patients do not take osteoporosis medication at all and yet others do not receive properly prescribed medications. In the most recent global review (124 studies) the prevalence of medication adherence varied between 12 and 95%. [32] Further, persistence and adherence to treatment decline over time even in RCT. [33] Clearly, suboptimal persistence and/or adherence to osteoporosis medication can reduce the treatment benefits of detaining loss or gaining BMD and increase the rik of fractures. [34] This was recently quantified by a large meta-analysis showing that fracture risk may increase by approximately 30% with both non-adherence and non-persistence to oral bisphosphonates. [35] Yet another problem is early treatment cessation that can lead to reversal of the effect of medication on BMD and even on fracture prevention. This holds for most anti-resorption treatments but has been particularly observed after suspending denosumab, a modern anti-resorptive compound mimicking the action of endogenous osteoprotegerin. Thus, current practice recommends switching to other osteoporosis treatment if denosumab is discontinued. [36] Overall, we should be viewing the future of our patients with osteoporosis with optimism, because we now have several drugs that can substantially reduce fracture incidence, by as much as 70% in the case of vertebral fractures and 30% in case of non-vertebral fractures. In addition, liaison services also have a promising impact on improving fracture prevention and decreasing the treatment gap. Liaison service represents a coordinated system by which experienced health professionals (a nurse practitioner, physician assistant, nurse or other professionals) ensure that individuals who suffer a fracture receive appropriate diagnosis, treatment and support soon after. [37] Future focus needs to be placed on the successful implementation in clinical practice of strategies to improve the assessment of treatment indication, adherence and, when fractures are unavoidable, liaising with proper strategies directed at minimizing the risk of future fractures. Last but not least, the recent advances in the genomics of osteoporosis will bring us a step closer to precision medicine i.e., gene-based individualized assessment of fracture risk and osteoporosis personalized therapy (**discussed below**). [38]

Changing the Narrative of Calcium and Vitamin D Supplementation

The majority of individuals at increased fracture risk receive calcium and vitamin D supplementation as prevention treatment. However, there have been emerging evidence from randomised controlled trials (RCT) showing that vitamin D supplementation alone or in combination with calcium does not reduce fracture incidence among community-dwelling older adults. [39,40] High dosages of vitamin D (>2000 IU/day) have also been ineffective and have even led to increased fracture risk mediated by potential increased propensity to fall. [41] Several aspects of my work included a Mendelian Randomisation (MR) approach (**discussed below**), which is analogous to RCT, allowing to infer causality between determinants and outcomes in a largely unconfounded setting. Using such MR approach, I showed that genetic predisposition to higher levels of serum vitamin D are not causally associated with increased BMD or a decrease in fracture risk (**Chapter 2.2.4**). Similarly, using the MR approach employing as instrument a Lactose intolerance genetic marker i.e., a proxy for (avoidance of) calcium intake, I showed that genetically determined increases in calcium levels have as well no effect on fracture risk. In subsequent recent work, we could confirm this contention using a genetic instrument of calcium serum levels (instead of calcium intake avoidance). [42] In both our studies, we assumed a linear effect of vitamin D or calcium on fracture risk. Future studies should assess possible threshold effects representing the true vitamin D deficiency state. The benefit of vitamin D in treating severe vitamin D deficiencies that lead to increased fracture risk and or severe bone malformations such osteomalacia and rickets is undisputable. Thus, supplementation is compulsory for individuals with severe low vitamin D or calcium levels, e.g., like institucionalized elderly individuals, while supplementation in individuals with adequate levels should be avoided. Similarly, children or adolescents with vitamin D deficiency may be at risk of not reaching maximum peak bone mass. [43,44] Moreover, vitamin D and calcium supplementation have been also related to adverse outcomes, such as falls [41] and increased risk of myocardial infarction. [45] Recenty it has been shown using the MR approach that the majority of the observational effects on vitamin D are due to the fact that the diseases or traits have causal effect on vitamin D contentrations and not other way around.[46] Overall, the general practitioner, endocrinologist or the orthopaedic surgeon who will treat an individual at increased fracture risk should seek treatment alternatives in individuals with normal vitamin D and calcium levels, that have a greater likelihood of being effective.

THERE IS MORE TO BONE THAN MEETS THE EYE

Although bone seems to be a very static organ, the bone tissue itself is dynamic because of the constant remodelling resulting from osteoblast and osteoclast activity, resulting in a panoply of enzymes, proteins, and by-products that are released in the circulation. Moreover, bone is an important regulator of calcium and phosphate metabolism, acts as a storage for many minerals, and is involed in maintaining the acid-base balance (buffering metabolic acidosis). [47] All these metabolic functions have been extensively studied in the past decade. One bone-active factor that demands further investigation is ***osteocalcin***; which has been implicated in a variety of physiological processes, namely glucose homeostasis, [48] brain development, [49] cognition, [50] and male fertility. [51] Although osteocalcin has been proposed to regulate bone formation, mice models have demonstrated very minor effects of osteocalcin on bone density and mineralization. [52] Interestingly, the extra-skeletal effects have been very prominent in mice such as increased adoposty, glucose intolerance and insulin resistance in oc $^{-/-}$ mice. [48,53] At the same time, there is an unmet need to translate the mice findings to humans given the potential health benefits of osteocalcin. Yet, before such clinical translation is made possible, we need to establish what is the active form of osteocalcin in humans. Osteocalcin exists in two forms in the circulation: ***carboxylated*** and ***undercarboxylated***. The latter lacks γ-carboxylation at one or more sites. In mice models, the undercarboxylated osteocalin is considered to be the active form of the molecule. However, we are lacking information from human studies as currently the majority of studies have measured only total serum osteocalcin levels. In addition, osteocalcin levels have been shown to have a U shape across the life course (highest in early adulthood, lower in mid-life, and then high again in older adults). [54] We observed a stronge inverse association between osteocalcin BMD and BMI (**Chapter 2.1.3**) which later we showed not to be causal (**Chapter 2.2.2**). Osteocalcin had a bidirectional causal association with femoral neck BMD; indicating that it can serve as a good marker of bone turover but it does not provied strong evidence if osteocalcin has a direct effects on bone. Overall, future studies examining the skeletal and extra skeletal-effects of osteocalcin should: i) assess both carboxylated and undercarboxylated osteocalcin; ii) evaluate longitudinal effects; and iii) estimate the effects across different age ranges.

INSIGHTS INTO THE DETERMINANTS OF MUSCULOSKELETAL HEALTH

The loss of bone and muscle with aging is bound to happen. However, the degree of bone weakening and rate of muscle loss, together with its clinical consequences are something we can modulate by identifying specific risk factors and subsequent interventions. Therefore, in this thesis, I examined both genetic (**Chapter 2 and Chapter 4**) and environmental factors (**Chapter 2 and Chapter 3**) that may affect musculoskeletal health and influence disease processes.

The role of environmental factors

The age-related loss of bone and lean mass, together with its function can be aggravated by a variety of lifestyle factors affecting one or both tissues, such as physical activity and nutrient intake. Given that osteoporosis and sarcopenia share many ***modifiable environmental factors,*** we can ***kill two birds with one stone***. Importantly, the environment plays an essential role in bone and lean mass accrual and their maintenance throughout life. For instance, failure to reach an optimal peak bone mass (PBM) before the age of 30 may substantially increase the risk of osteoporosis and fragility fractures later in life. [55] It has been estimated that a 10% increase in PBM would delay the onset of osteoporosis by an average of 13 years. [56] Moreover, the structural and biomechanical properties of the bone acquired during the first three decades of life are also related with increased fracture risk in older adults. [57] Therefore, aiming to optimize reaching a high PBM should be the first step towards improving bone health in adults and elderly people. The advances in the field of paediatric bone health over the recent years have brought many insights into the clinical determinates of PBM. [58] Several important modifiable factors, such as nutrition, hormonal status, and physical activity, can influence bone acquisition in children. Children's parents and caregivers i.e, paediatricians need to be well informed regarding all these factors in order to maximize PBM attainment. Then, after the age of 35 years, there is a gradual loss of bone and lean mass without any manifestation until an ominous clinical endpoint occurs, such as fracture. A multitude of clinical, lifestyle and environmental factors can accelerate the loss of bone and muscle mass. Increasing awareness about predisposing risk factors and prevention strategies among the general population and the general practitioners, will have tremendous impact on the primary prevention of osteoporosis and sarcopenia. Similarly, educating the general population on proper nutrition (increased protein intake, avoidance of alcohol, antioxidant rich food) and/or adequate physical activity will very likely improve the musculoskeletal health of young adults and later in their life as well. The age-related decline in lean mass could be easily reverted and even increased using well-structured and detailed exercise

regimes. However, only increasing lean mass is not sufficient; changes in mass need to be also followed by an increase in muscle strength and power as well. Recently, the National Institute on Aging (NIA) launched an exercise and physical activity campaign called ***Go4Life*** to encourage older people to be active in order to prevent osteoporosis, reduce falls and fracture risk among other chronic conditions. This program contains well-curated evidence-based exercises for increase of endurance, strength, balance, and flexibility. The exercises are easy to follow and are accommodated to individuals with specific chronic conditions. Overall, environmental factors have a large impact on musculoskeletal health and by intervening on them from a young age, we can successfully delay/prevent the onset of osteoporosis and sarcopenia later in life. Notably, even in individuals with higher genetic predisposition for osteoporosis or sarcopenia, lifestyle and environmental modifications can reduce disease risk notably.

The role of genetic factors

Positive family history is an independent risk factor for many medical conditions, importantly among which fragility fractures. Unravelling the genetic underpinnings of fracture risk could considerably improve the diagnostic and risk stratification accuracy. However, several challenges are impeding genetic discoveries underlying fracture risk, such as: a) ***phenotypic and genotypic heterogeneity*** – a variety of factors and pathways can all lead to fracture alone or in combination; b) ***information bias*** – when fractures are collected retrospectively using a variety of questionnaires or lacking validation; ***c) time-to-event*** – fractures occur later in life and we need a considerable follow-up time to capture their occurrence; and **d)** ***case definition*** –majority of high-trauma fracture cases are considered to occur in individuals without a diagnosis of osteoporosis and in the presense of normal BMD. With regard to the latter, high-trauma fractures have been systematically exluded from any observational studies or clinical trials. Nevertheless, hight-trauma fracture can also be associated with low BMD and increase the risk of future fracture in eldery people. [59] Some methods to overcome these limitations include increasing the discovery sample size and/or by using instead so-called "endophenotypes". What can be a better endophenotype for osteoporosis and fracture risk than BMD? As discussed above, low BMD is a strong and well-established risk factor for fractures. Further, BMD is widely available as is used in clinical practice to diagnose osteoporosis. BMD also constitutes a very precise measurement with stable standard deviations across devices and geographical regions allowing realiable comparisons between studies. Last but not least, BMD is a highly heritable trait picking up very well "true" bone biology, altogether representing an extremely good endophenotype. GWAS on BMD alone have yielded an outstanding amount of discoveries along the past decade identifying many variants pointing to genes involved in relevant bone pathways (mesenchymal stem cell differentiation

or WNT signalling among many others) but also novel unexplored ones (like oncogenic pathways and melanogenesis) (reviewed in **Chapter 2.2.1** and **Chapter 4.1**). [60,61] Nevertheless, DXA-based areal BMD measurements hold limitations as they cannot provide information on volumetric BMD, trabecular or cortical density nor microarchitecture. Thus, there might be other components to assess fracture risk beyond BMD that are yet to be discovered. Until today, there are no known genes affecting fracture risk independently of BMD. Nonetheless, I observed a moderate genetic correlation between risk of falling and fracture risk (**Chapter 4.3**) which can reflect that some genes that predispose people to fall may also increase fracture risk. While all known fracture loci are also BMD loci, we did not observe any genetic correlation between falls and BMD. This implies that low BMD and increased falls risk might have different biological mechanisms emerging towards the same endpoint i.e., fracture. Despite the null genetic correlation, falls and BMD might still hold shared influence on some comorbidities. For example, a variety of medications can increase the propensity to fall on one hand, while weakening our bones on the other hand (e.g. glucocorticosteroids). Thus, depicting the genetic architecture of the medication-induced falls and/or medication-induced bone loss is a promising future step to understand the underlying biology of fracture risk. Finally, BMD is a necessary but not necessarily a sufficient factor leading to fracture. Fracture risk at the end will depend on bone mass, architecture, strength, and quality in relation to the response to forces applied to it.

Lean mass and handgrip strength can also be good endophenotypes to investigate the genetic landscape of sarcopenia, and risk of falling and fractures. However, genetic studies on muscle mass have lagged behind BMD discoveries, and only few GWASs have been conducted so far. In the first GWAS meta-analysis on lean mass, which was measured by DXA or bioimpedance (BIA) and adjusted for sex, age, height, and fat mass, only five loci incuding 2q36.4 *(IRS1),* 4q22.1 (H*SD17B11),* 5q14.2 *(VCAN),* 15q25.2 *(ADAMTSL3),* and 16q12.2 *(FTO*) were identified despite a large sample size (n=80,652). [62] Although both DXA and BIA techniques show relatively high correlation, [63] they still might be assessing different biological properties of lean mass. In a recent large-scale study from the UK Biobank (450,580 individuals), in total 561 loci were identified as associatied with BIA-derived appendicular lean mass explaining ~11% of the phenotypic variance. [64] Further investigations directed at establishing the different yield in genetic discoveries between DXA and BIA assesments of lean means are warranted. Genetic variants have been also associated with measures of muscle function. For example, we performed the first GWAS on handgrip strength, using muscle strength as a marker of muscle performance, identifying 16 variants (*POLD3, TGFA, ERP27, HOXB3, GLIS1, PEX14, MGMT, LRPPRC, SYT1, GBF1, KANSL1, SLC8A1, IGSF9B, ACTG1, DEC1, and HLA*). [65] Recently, in much larger well-powered study settings (comprising over 330,000 individuals) the number of known loci associated with handgrip

strength surpassed 100 and showed an important role of the central nervous system in strength performance. [66] Overall, the past five years have been quite important for muscle research and I expect in the next five years for us to examine in more depth the biological role of all these novel discoveries using human-cells and animal models.

One gene, one disorder or one gene, multiple disorders?

Lean mass and BMD hold high phenotypic (~0.40)[67] and genetic correlation (~0.50). [68] The genetic correlation refers to the shared heritability between these traits and it can be an indication of the presence of genes that affect both BMD and lean mass variation i.e., pleiotropy. In **Chapter 4.1,** I reviewed the existing literature on GWAS studies for both traits and found several cross-phenotypic correlations. [61] Currently, in the NIHGR GWAS catalogue, around 44% of the reported genes are associated with two or more traits. [69] Recently, 341 loci were reported as pleiotropic across 42 traits; [70] which is a large number among the tested diseases. In the next years, more GWAS will emerge and the number of shared loci is expected to increase accordingly.

Pleiotropy can be classified as: **a)** ***genome-wide,*** **b)** ***regional,*** and **c)** ***single variant pleiotropy***. Currently, there are a multitude of methods developed to assess pleiotropy. They can be classified into ***univariate*** and ***multivariate***. Univariate methods utilize summary statistics data of singe-trait GWAS, whereas, multivariate methods require individual level data. Similarly, pleiotropy can be assessed at genome-wide level using polygenic risk scores (PRSs) to test if variants associated with one trait/disease explain a significant proportion of the variance of a different trait/disease. Moreover, genetic correlations have been widely used as an indicator of pleiotropy. Multivariate methods for assessing genetic correlation include tools such as GCTA[71] and BOLT-REML. [72] Nowadays, we can also estimate the genetic correlation between traits by simply using summary statistics as implemented in linkage disequilibrium score regression (LDSR or LDSC). [73] As described above, genetic correlation is only a general indication of pleiotropy between traits. Therefore, we can narrow the search to a specific region, i.e., regional pleiotropy. Univariate approaches for assessment of regional pleiotropy include pleiotropic region identification method (PRIMe) [74] and GWAS-pw, [70] whereas, multivariate approaches include canonical correlation analysis metaCCA [75] and mtSET [76] among others. Last but not least, a single point method for determining pleiotropy has been incorporated in many tools such as cross-phenotype meta-analysis (CPMA), [77] ASSET, [78] MultiMeta [79] and conditional false discovery rate (cFDR). [80] Recently, a new method has been developed named multi-trait analysis of GWAS (MTAG). [81] This method is a generalization of the inverse-variance weighted meta-analysis and provides adjusted effect estimates for all included traits taking into account the genetic correlation between them. All of the above methods and tools have been successfully reviewed and discussed elsewhere. [82,83,84] The majority

of GWAS data has been made publically available, facilitating the search of cross-phenotype associations. Nowadays, browsing GWAS results has been facilitated with the development of the ***GWAS Atlas,*** which at the moment of writing the database contained 4,756 GWAS from 473 unique studies across 3,302 unique traits and 28 domains. [85] On this platform, we can also obtain estimates of genetic correlation between multitudes of traits. Furthermore, we can also browse phenome-wide association results (PheWAS). This approach is similar to GWAS but it relates a selected SNP with a multitude of traits i.e., the phenome scan. The musculoskeletal community is currently developing the ***Musculoskeletal Knowledge Portal*** (http://www.mskkp.org/) which constitutes a genomic data mining platform aimed at accelerating discoveries for musculoskeletal traits and diseases; enabling browsing, searching, and analysing human genetic and genomic information linked to functional assesments relevant to musculoskeletal biology. Importantly, this web-based tool is very well suited to study complex pleiotropic relationships, i.e., identifying antagonic effects across traits and diseases. For instance, some *SREBF1* variants have shown to increase BMD and decrease lean mass. [68] Yet, it is important to remember that all the above statistical approaches only provide evidence of pleiotropy but they do not provide information on the underlying biological mechanisms, which need to be delineated by follow-up functional studies. Information on pleiotropic gene effects will have important clinical value especially in drug repurposing efforts or in situations where a gene-drug treatment is beneficial for one trait but detrimental for another, i.e., pinpointing adverse effects.

CLINICAL TRANSLATION OF GWAS DISCOVERIES

GWAS have helped us to gain insight into the genetic landscape of many traits and diseases that have had vast practical implications such as:

i. **Predicting individual risks of disease** using genetic risk scores (GRS), which are simply defined as a sum of genetic variants associated with a specific trait/disease. The higher the number of deleterious alleles the higher the score. GRSs follow a normal distribution of disease risk implying that most individuals will have an average number of risk alleles, while at both ends of the distribution (left and right) there will be a cluster of individuals with very low or very high disease risk alleles. This is in principle a proof of concept that genetic information can be utilised in non-genetic disease risk prediction. Many successes and failures have followed the early stages of incorporating genetics into prediction models. Nowadays, GRSs have achieved great successes in relationship with many complex diseases such as diabetes, [86,87] coronary artery disease ,[88] and depression.[89] In addition, Khera *et al.* have

shown that genome-wide polygenic scores for complex diseases such as coronary artery disease, atrial fibrillation, type 2 diabetes, inflammatory bowel disease, and breast cancer can identify individuals with a risk equivalent to that of monogenic mutations. [87] Despite early discouraging results of GRSs in relationship with fracture risk (i.e. low prediction accuracy),[90] drastic improvement in the prediction modelling have been achieved with novel genetic discoveries and improved statistical modelling [91] and I expect many more to follow. Currently, there is some scepticism regarding the clinical value of the GRS approach. Nevertheless, genetics forms part of a person's disease profile and represents the earliest measurable disease risk factor; thus, I firmly believe a PRS can be quite informative by adding value over and above clinical risk factors. Besides identifying people at the highest risk categories, GRSs can also help in identifying people at risk for disease progression or complications.[92,93] Genetics can contribute to improve treatment strategies by identifying people who will most benefit from treatment or are at higher risk of adverse effects. [94] Importantly, given that genetic effects are stable across the life-course, we may be also able to predict the onset of disease decades before it occurs. On the other hand, low risk re-assurance is another important and often neglected use embedded in the PRS risk gradient; this can for example be used to avoid performing unnecessary (and usually expensive) diagnostic workup or interventions in people who do not need them. Altogether, I believe in no time genetic information will be incorporated in the clinical guidelines to prompt medical decision-making.

ii. **Strengthen causal inference in observational research:** Besides disease prediction and risk stratification the use of GRSs has been further expanded into linking traits and diseases to provide more robust evidence of causality. As we discussed above, RCTs are the golden standard in testing whether exposure is causal for a specific outcome. However, it is not always feasible to conduct an RCT and alternatives have been sought. As our genotypes are randomised by nature at conception (Mendel's second law of independent assortment), the MR approach has been developed to derive more robust evidence of causal associations. I describe the MR approach in detail in **Chapter 2.2.5**. Shortly, MR uses genetic variants that are fixed after conception as instrumental variables for modifiable risk factors to derived un-confounded causal estimates. Several assumptions of MR need to be satisfied in order the estimates to be valid (see **Chapter 2.2.5** and **Box 1** in the introduction section). Running an MR analysis prior to a RCT can be helpful to foresee adverse effects and or expected outcomes. One pitfall of the MR studies is that only test for linear relationships between the exposure and outcome.

iii. **Dissect genetic association signals through deep phenotyping** can help us gain additional insight into the underlying biology of complex traits and diseases. Performing deep phenotyping in extremely large cohort studies is impractical and

expensive. [95] Thus, observational studies have opted to do deep phenotyping in smaller sample sets selected based on the extremes of the phenotypic distribution of the trait of interest. However, selecting groups of interests from truncate trait distributions from measured factors in observational studies can be easily confounded and result in biased selections, that end up sacrificing power. We can follow a similar approach but then leveraging genetic information, which, as we discussed above, should be un-confounded under the principle of Mendelian randomization. This approach, referred to as Recall by genotype (RbG), [95] is defined as a prospective recruit of individuals with extremely low and high genetic predisposition for a specific trait or disease. The efficacy of the design will depend on the trait under investigation, sample size, the study design, how the variants were selected, and unbalanced loss to follow-up by genotype. Thus, we should carefully assess all these factors while framing our RBG study.

iv. **Reveal potential drug targets:** GWAS studies have been successful in rediscovering known drug targets and have underlined potential novel drug targets. Up to 2,205 of the 20,300 protein-coding genes annotated in Ensemble version 73 have been drugged or are druggable; [96] mapping GWAS findings to these drugggable proteins can facilitate drug target identification in relation to a specific trait of interest. Selecting genetically supported drug targets could double the success rate in clinical development. [97] Further, genomics can facilitate pinpointing medications subjet to drug repurposing, i.e., a compound that has already being subject to the long-lasting and very expensive process of approval can be identified as indicated for another indication, distinct but equally (or more) effective than the original purpose for which it was developed. [98]

PRACTICAL LIMITATIONS OF GWAS OF MUSCULOSKELETAL TRAITS AND BEYOND

"But, alas, that which glitters in not always gold"

Despite large successes in the past decades, there are still several limitations to GWAS, that we need to acknowledge related to the trait under investigation or the discoveries:

i. ***Trait heterogeneity:*** Phenotypic misclassification is a problem in many case-control studies that can reduce the power to detect association. [99,100] It has been suggested that heterogeneity of 50% requires three times larger sample size as compared to a scenario of no heterogeneity. [101] Heterogeneity may also affect the heritability estimates and the trait variance explained by genetic variants. In addition, heterogeneity can lead to an underestimation of the effect estimates identified by GWAS.

Heterogeneity is common in the association analysis of complex traits, and to reduce it the two main approaches consist of either a) selecting homogeneous subgroups of the study population in the analysis (sacrificing power); or b) increase sample size (typically at expense of phenotype definition). In our studies, both falling risk and fracture risk constitute highly heterogeneous traits. Instead of doing sub-group analysis across homogeneous groups, we conducted our studies in extremely powerful study settings that helped overcome the high heterogeneity of these two traits and provided adequate power to detect true GWAS signals.

ii. ***Tagging true causal variants:*** The large-scale discoveries have made it difficult for us to keep up the transition from GWAS to function. In order to establish the function, we need to delineate first the right causal genes, a path that has not been well-paved in the past but which is now critical to the present. Tagging the underlying true causal variants has been difficult due to the strong LD existing between the most significant disease-associated variant and the co-inherited variants. In addition, a large proportion (>80%) of the genome-wide significant (GWS) variants are located in non-protein-coding regions; making the follow-up analysis even more difficult given the ill-defined regulatory regions of genes. In addition, the relevance of function is not always clear with respect to target tissues. Currently, the search for causal genes involves creating credible sets of SNPs using Bayesian approaches, fine-mapping these sets to functional elements such as eQTLs facilitated by the Encyclopedia of DNA Elements (ENCODE) project, [102] the NIH Roadmap Epigenomics Consortium, [103] the FANTOM5 project, [104] among others, that altogether encompass several hundred human cell types and tissues created to facilitate the (epi)genomic annotations. The eQTL approach has been fruitful for many traits, however, given the lack of bone and muscle tissues in publicly available databases, not much progress has been made in relation to musculoskeletal outcomes. Therefore, creating bone- or muscle-specific eQTL resources can facilitate the post-GWAS analyses, providing more insight into the disease processes underlying osteoporosis and sarcopenia. While eQTLs can be informative, they are quite ubiquitous (unespecific) and constitute only a small piece of the biological puzzle. Thus, conclusions regarding causality of GWAS-identified variants should not be based solely on one level of evidence. Combining evidence from DNA methylation (mQTLs), DNase hypersensitivity (dsQTLs), TF binding (bQTLs) and histone modification marks associated with regulatory elements will increase the degree of evidence for functional implication of the candidate causal variants. [105] In **Chapter 2.2.1,** we discuss a comprehensive target gene identification pipeline for functional testing we developed in Morris et al.[106] to prioritize genes for heel BMD associated loci. The following steps are embedded in the pipeline: (a) identifying genes most proximal to the fine-mapped SNPs; b) identifying genes containing fine-mapped SNPs overlapping their gene

region; (c) determine genes containing fine-mapped SNPs coding variants; (d) assess genes identified to be in 3D contact with fine-mapped SNPs in human osteoblasts or osteocytes through high-throughput chromatin conformation capture (Hi-C) experiments; (e) establishing the closest gene to fine-mapped SNPs also mapping to ATAC-seq (**A**ssay for **T**ransposase-**A**ccessible **C**hromatin using sequencing) peaks in SaOS-2 (Sarcoma osteogenic) cells; and (f) verify genes within 100 kbp of fine-mapped SNPs. Overall, combining and integrating multiple functional annotation tools will increase the likelihood for identifying the true causal gene.

iii. ***Analytical constraints:*** To date DXA images have been used to measure areal BMD, TBS, and hip shape. However, there are many additional features in the images that can provide valuable information that may improve fracture prediction. Recent advances in medical image analysis aided by various **artificial intelligence (AI)** approaches, can help to establish and derive patterns from the medical images that can discriminate between groups, such as images from individuals with or without a certain disease. Machine learning and deep learning are the two most trending AI approaches in medical image analyses although they differ in their capabilities and implications. **Machine learning** deals with simpler algorithms and operations and it typically requires guidance i.e., structured/labelled data. On the other hand, **deep learning** is a subset of machine learning, for which we don't really need a structured/labelled data (unsupervised). In deep learning between the input (what we add) and output layer (what we get) there are multiple hidden layers so-called artificial neural networks, which will do all the work for us; each hidden layer will define a specific feature of the image that will pass it on the next layer and so on until a "*decision"* is made. Deep learning is highly accurate in the presence of a large amount of data at the expense of very large computational power. Both approaches require numerous data training sessions in order to learn enough to provide reliable and accurate results. Overall, "*machine learning makes informed decisions based on what it has learned based on the features we define, whereas, deep learning creates a network that can learn and make an intelligent decision based on the features it creates on its own*". [107] Such artificial intelligence approaches are not confined to the analysis of phenotypes, but are also emerging as viable alternatives to model the complexity of genomic data; serving as data reduction approaches that do not require sacrificing information while maximizing the value of information hidden or awaiting interpretation across multiple layers of Big Data i.e., multi-omics integration.

iv. ***Generalizability and health disparities:*** Population stratification, a state where sub-populations are distinguishable by observed genotypes and differences in phenotype distribution, have been shown to lead to false positive or negative associations between a genotype and a trait. [108] In order to minimise this effect, GWA

studies in the past have focused on running analysis in ethnically homogeneous groups, e.g. European or Asian ancestry only. While effective for their original purpose, this has led to over-representation of participants of European ancestry in GWAS research which account for up to 80% of all GWAS samples. [109] This is quite relevant for clinical practice as information arising from a single ancestral group may not be applicable for the other ancestral groups and can affect the accuracy of the genetic prediction models across different populations. [109,110] For example, pathogenic variants associated with hypertrophic cardiomyopathy in the American white population have been shown to be benign for the American African ancestry population what has led to misclassification of benign variants as pathogenic in this population in clinical practice. [111] Overall, the predictive ability of European ancestry-derived PRS is lower in non-European samples for certain diseases; [110] particularly for African samples where the linkage disequilibrium (LD) structure is highly fragmented (reduced LD and smaller haplotypes) compared to Europeans and thus, the true causal variants would be less likely captured. European derived PRSs are approximately one-third as informative for African individuals, as they are for European individuals. [110] These discrepancies can be the consequence of several factors, such as a difference in allele frequencies, LD and dissimilar genetic architecture (derived from differing environmental influences) across populations. This represents major ethical and scientific challenges surrounding clinical translation and, at present, the most critical limitation to genetics in precision medicine. [109] Introducing diversity in genetic studies has been a top priority of the research community in the past few years. Studies including non-European populations are expected to uncover novel genotype-phenotype associations that can boost the predictive value of PRS in clinical practice. Last but not least, it is very important to mention that the abovementioned challenges are not applicable for all traits and diseases.

WHAT DOES THE FUTURE HOLD FOR GENETICS?

Multiple efforts to date have made significant contribution to the understanding of the genetic architecture of the musculoskeletal system. The term ***genetic architecture*** refers to the genetic factors responsible for the heritable phenotypic variation of any trait or disease; [112] succinctly defined by the number of genetic loci affecting a trait, their effect sizes and frequencies, and their interactions with other genes or environmental factors. [113] Establishing the genetic architecture of a trait or disease is crucial as it will provide the insight needed to choose study populations, study designs and technological approaches best suited for the successful identification of

underlying gene variants. In a perfect world, a scenario with accurate phenotyping, comprehensive scrutiny of genomic variation and sufficiently powered study populations will fully unveil the genetic architecture of trait or dieases. [114] As we are not there yet, there is still lot of work to be done. For start, the era of ***Big Data*** has led to remarkable breakthroughs in the field of genomics and will continue to do so with ever increasing sample size of GWAS and other types of genetic and non-genetic studies emerging in the upcoming years. Eventually, we will reach such large sample sizes that no novel loci will remain to be discovered, filling-in the knowledge gaps in the genetic architecture of many traits and diseases. Currently, in the bone field, the maximum sample size we have reached is half a million individuals with GWS SNPs explaining up to 20% of the variance of estimated heel BMD. Henceforth, forming new large-scale biobanks or combing efforts across different existing biobanks such as the UKBB, 23andMe and the Million Veterans Program (MVP) will further push the boundaries of our knowledge in the complex landscape of osteoporosis and sarcopenia. Moreover, there is also an ongoing effort to collect data across the world and create a global public genome, health and trait database initiated by The Global Network of Personal Genome Projects. [115,116,117] Nevertheless, a cautious interpretation of results from the large biobanks may be needed as they can be susceptible to selection bias and may not be representative for all groups of people. For instance, in the UKBB only 5% of the total invited individuals (9 million) have responded and are part of the study and this may lead to selection bias. [118] This has been recently evidenced by the identification of numerous artefactual associations with sex across the autosomes, appearing as result from differential participant response across sexes. [119] Alongside sample size increments, the momentum Big Data is bringing can be also attributed to the rise of affordable NGS technologies, for ***targeted sequencing whole-exome sequencing (WES), whole-genome sequencing (WGS).*** Not long ago, it would take years to sequence the whole genome (all 3 billion base pairs) at a very high cost, while nowadays, we can do it within a single day for less than 1,000 Euros. However, information about its applicability in clinical settings and the broader population is yet to be determined. Recently a small pilot study was set up in ordered to determine the risk and benefits of integrating WGS into primary care. [120] Nine primary care physicians (PCPs) and 100 of their healthy patients were enrolled in this study. The patients later were randomized into receiving a family history report alone or in combination with an interpreted WGS report. Overall, one out of five health patients that were sequenced had previously unrecognized rare variants with potential risk for Mendelian disease, whereas, one out of 25 had clinically confirmed abnormalities that prompted clinical actions that would have not been taken without genome sequencing. [120] In the bone field, combining WGS and imputed GWAS data has yielded several rare variants (MAF<0.05) in relationship with BMD and fracture risk. [121,122,123] While 15 years ago

it was unimaginable to genotype a few hundred SNPs in a larger number of people, currently the same applies for performing WGS. The costs of WGS are expected to fall further with time, actually now making the costs of data storage the bottle neck for even more affordable costs. Nevertheless, I expect WGS to be embedded in the future of genetics and believe it will definitely transform personalized medicine by detecting ultra-rare and individual-specific genetic variations which play a role on disease. Although, current imputation methods provide reliable estimates and approximate the power from WGS to assess less-frequent and even rare variants, ultra-rare variants with large effects and unmeasurable clinical implications cannot be imputed as they lack LD. Rare variants with MAF<0.01 and low LD metrics can account for up to 40% of the phenotypic variance for height and can only be reliably detected using WGS. [124] Such increase in variance explained can substantially improve the GRSs prediction accuracy for many complex traits. [124] Another benefit of WGS is that we can study the burden of rare variants across different functional elements that cannot be assessed with ordinary GWAS. Large biobanks with genotyping arrays in combination with WGS will be important for gene discovery and or gene predictions in the following years. With limitations in mind, an alternative to WGS in the clinic, which can be both beneficial and cost-effective is the use of genotyping arrays. While rare variants not sitting on the array will be missed, fast and cheap (down to <30 euros per DNA sample) screens can be made on patients for a large number of selected DNA variations. This is the core goal of the ***Genotyping on ALL patients (GOALL) project*** lead by an excellent team of researchers' part of Erasmus MC. The vision of this project is that all patients that come into Erasmus MC be genotyped in order to use this information to improve their diagnosis and treatment.

Next, there is also a scientific wave moving toward the ***multi-omics approach***. However, as compared to GWAS, the other -omics approaches, such as ***epigenomics*** or ***microbiomics*** have yielded less findings due to several factors such as lack of trait-specific tissues, large costs and/or low power. Specific to the musculoskeletal field, we can expect that changes in blood methylation can be a good proxy of methylation status of bone considering that osteoclasts and monocyte/macrophages share the same stem cell precursors. However, in our large-scale EWAS study (N_{max}=5,515) we observed at most small effects of methylation changes in whole blood on BMD. [125] In contrast, DNA methylation studies (N_{max}=84) using bone biopsies have shown a significant difference in methylation levels between healthy and osteoporotic women. [126] Future well-powered efforts performing targeted EWAS of specific blood cell types with clear role in bone biology or in bone cells may be more informative of the epigenetic changes occurring in the bone tissue. On yet another -omic layer, there are several ongoing efforts seeking to characterize the association of the gut microbiome with different musculoskeletal outcomes. It has been suggested that the

gut microbiome can affect bone health by alterations in the immune system leading to defected osteoclasts activity. [127] On the other hand, oral probiotics, commercially prepared substances of living microorganisms with positive health benefits, have been associated with increased trabecular bone volume in mice and reduced bone loss. Impressively, similar effects have been observed in postmenopausal women. [128] Assesing the mice findings in humans can provide additional understanding of disease mechanisms.

PERSONALIZED MEDICINE: THE FUTURE IS NOW

My research is a small contribution to a larger cause, i.e., genomic medicine. In the following decades, we will successfully integrate genomic and clinical data to support clinical decision-making at all three levels of musculoskeletal disease prevention i.e., primary, secondary and tertiary. Genetic testing before birth or at any time during a person's life will be integrated into the existing clinical workflow and will improve the identification of people at risk of osteoporosis, sarcopenia and fracture risk (primary care) by aiding existing clinical algorithms. Next, genomics will drastically improve the diagnostic accuracy and therapeutic efficacy for many musculoskeletal outcomes (secondary care). Furthermore, genetics will also help to identify individuals with highest risk of disease progression and/or of severe complications (tertiary care). Most importantly, the diagnosis and treatment will be tailored for each individual patient based on his/hers genetic makeup. However, genetics will not be the *ultimate element* of personalized medicine. In the long run, information from the other omics fields such as epigenomics, transcriptomics, proteomics and metabolomics will also find their place in personalized medicine. This will result in a large influx of data that we would not be able to handle on our own, but there is no need to worry, as artificial intelligence has emerged to stay. *Summa summarum*, incorporating genetic information into disease risk prediction and prevention will be a big step for one person and a giant leap for personalized medicine.

REFERENCES

1. Hernlund, E. et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. Arch. Osteoporos. 8, 136 (2013).
2. Trajanoska, K. et al. Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. Bone 114, 116–124 (2018).
3. Farmer, M. E., White, L. R., Brody, J. A. & Bailey, K. R. Race and sex differences in hip fracture incidence. Am. J. Public Health 74, 1374–80 (1984).
4. Cauley, J. A. Defining ethnic and racial differences in osteoporosis and fragility fractures. in Clinical Orthopaedics and Related Research vol. 469 1891–1899 (Springer New York LLC, 2011).
5. Curtis, E. M. et al. Epidemiology of fractures in the United Kingdom 1988-2012: Variation with age, sex, geography, ethnicity and socioeconomic status. Bone 87, 19–26 (2016).
6. Lewiecki, E. M. et al. Hip fracture trends in the United States, 2002 to 2015. Osteoporos. Int. 29, 717–722 (2018).
7. Icks, A. et al. Incidence of hip fractures in Germany, 1995-2010. Arch. Osteoporos. 8, 140 (2013).
8. Amin, S. C. M., Achenbach, S. J., Atkinson, E. J., Khosla, S. & Melton Iii, L. J. Trends in Fracture Incidence: A Population-Based Study Over 20 Years. J Bone Min. Res 29, 581–589 (2014).
9. Cassell, E. & Clapperton, A. A decreasing trend in fall-related hip fracture incidence in Victoria, Australia. Osteoporos. Int. 24, 99–109 (2013).
10. Korhonen, N. et al. Continuous decline in incidence of hip fracture: nationwide statistics from Finland between 1970 and 2010. Osteoporos. Int. 24, 1599–603 (2013).
11. Gullberg, B., Johnell, O. & Kanis, J. A. World-wide projections for hip fracture. Osteoporos. Int. 7, 407–13 (1997).
12. Cummings, S. R. et al. BMD and risk of hip and nonvertebral fractures in older men: a prospective study and comparison with older women. J. Bone Miner. Res. 21, 1550–6 (2006).
13. Kanis, J. A. et al. Ten year probabilities of osteoporotic fractures according to BMD and diagnostic thresholds. Osteoporos. Int. 12, 989–995 (2001).
14. Nguyen, N. D., Frost, S. A., Center, J. R., Eisman, J. A. & Nguyen, T. V. Development of prognostic nomograms for individualizing 5-year and 10-year fracture risks. Osteoporos. Int. 19, 1431–1444 (2008).
15. Hippisley-Cox, J. & Coupland, C. Predicting risk of osteoporotic fracture in men and women in England and Wales: Prospective derivation and validation of QFractureScores. BMJ 339, 1291–1295 (2009).
16. Kanis, J. A., Johnell, O., Oden, A., Johansson, H. & McCloskey, E. FRAXTM and the assessment of fracture probability in men and women from the UK. Osteoporos. Int. 19, 385–397 (2008).
17. Edwards, M. H. et al. Clinical risk factors, bone density and fall history in the prediction of incident fracture among men and women. Bone 52, 541–547 (2013).
18. Trajanoska, K. et al. Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study. BMJ 362, k3225 (2018).
19. Moreland, J. D., Richardson, J. A., Goldsmith, C. H. & Clase, C. M. Muscle Weakness and Falls in Older Adults: A Systematic Review and Meta-Analysis. J. Am. Geriatr. Soc. 52, 1121–1129 (2004).

20. Harvey, N. C. et al. Appendicular lean mass and fracture risk assessment: implications for FRAX® and sarcopenia. Osteoporos. Int. 30, 537–539 (2019).
21. Harvey, N. C. et al. Measures of Physical Performance and Muscle Strength as Predictors of Fracture Risk Independent of FRAX, Falls, and aBMD: A Meta-Analysis of the Osteoporotic Fractures in Men (MrOS) Study. J. Bone Miner. Res. 33, 2150–2157 (2018).
22. Ammann, P. & Rizzoli, R. Bone strength and its determinants. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA vol. 14 Suppl 3 (2003).
23. Beck, T. J. Extending DXA beyond bone mineral density: Understanding hip structure analysis. Current Osteoporosis Reports vol. 5 49–55 (2007).
24. Samelson, E. J. et al. Cortical and trabecular bone microarchitecture as an independent predictor of incident fracture risk in older women and men in the Bone Microarchitecture International Consortium (BoMIC): a prospective study. lancet. Diabetes Endocrinol. 7, 34–43 (2019).
25. Leslie, W. D. et al. Risk-equivalent T-score adjustment for using lumbar spine trabecular bone score (TBS): the Manitoba BMD registry. Osteoporos. Int. 29, 751–758 (2018).
26. Shevroja, E. et al. Clinical Performance of the Updated Trabecular Bone Score (TBS) Algorithm, Which Accounts for the Soft Tissue Thickness: The OsteoLaus Study. J. Bone Miner. Res. (2019) doi:10.1002/jbmr.3851.
27. McCloskey, E. V. et al. A Meta-Analysis of Trabecular Bone Score in Fracture Risk Prediction and Its Relationship to FRAX. J. Bone Miner. Res. 31, 940–948 (2016).
28. Hernlund, E. et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch. Osteoporos. 8, 136 (2013).
29. Keshishian, A. et al. Examining the treatment gap and risk of subsequent fractures among females with a fragility fracture in the US Medicare population. Osteoporos. Int. 28, 2485–2494 (2017).
30. Hajcsar, E. E., Hawker, G. & Bogoch, E. R. Investigation and treatment of osteoporosis in patients with fragility fractures. CMAJ 163, 819–822 (2000).
31. Kanis, J. A., Svedbom, A., Harvey, N. & McCloskey, E. V. The Osteoporosis Treatment Gap. J. Bone Miner. Res. 29, 1926–1928 (2014).
32. Yeam, C. T. et al. A systematic review of factors affecting medication adherence among patients with osteoporosis. Osteoporosis International vol. 29 2623–2637 (2018).
33. Hiligsmann, M. et al. Determinants, consequences and potential solutions to poor adherence to anti-osteoporosis treatment: results of an expert group meeting organized by the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO) and the International Osteoporosis Foundation (IOF). Osteoporos. Int. 1–11 doi:10.1007/s00198-019-05104-5.
34. Siris, E. S. et al. Impact of Osteoporosis Treatment Adherence on Fracture Rates in North America and Europe. Am. J. Med. 122, S3 (2009).
35. Ross, S. et al. A meta-analysis of osteoporotic fracture risk with medication nonadherence. Value Heal. 14, 571–581 (2011).
36. McClung, M. R., Wagman, R. B., Miller, P. D., Wang, A. & Lewiecki, E. M. Observations following discontinuation of long-term denosumab therapy. Osteoporos. Int. 28, 1723–1732 (2017).

37. McLellan, A. R., Gallacher, S. J., Fraser, M. & McQuillian, C. The fracture liaison service: Success of a program for the evaluation and management of patients with osteoporotic fracture. Osteoporos. Int. 14, 1028–1034 (2003).
38. Nguyen, T. V. & Eisman, J. A. Pharmacogenetics and Pharmacogenomics of Osteoporosis: Personalized Medicine Outlook. in Genetics of Bone Biology and Skeletal Disease: Second Edition 139–157 (Elsevier Inc., 2018). doi:10.1016/B978-0-12-804182-6.00009-5.
39. Zhao, J.-G., Zeng, X.-T., Wang, J. & Liu, L. Association Between Calcium or Vitamin D Supplementation and Fracture Incidence in Community-Dwelling Older Adults. JAMA 318, 2466 (2017).
40. Bolland, M. J., Grey, A. & Avenell, A. Effects of vitamin D supplementation on musculoskeletal health: a systematic review, meta-analysis, and trial sequential analysis. Lancet Diabetes Endocrinol. 6, 847–858 (2018).
41. Sanders, K. M. et al. Annual High-Dose Oral Vitamin D and Falls and Fractures in Older Women. JAMA 303, 1815 (2010).
42. Cerani, A. et al. Genetic predisposition to increased serum calcium, bone mineral density, and fracture risk in individuals with normal calcium levels: Mendelian randomisation study. BMJ 366, (2019).
43. Lehtonen-Veromaa, M. K. et al. Vitamin D and attainment of peak bone mass among peripubertal Finnish girls: a 3-y prospective study. Am. J. Clin. Nutr. 76, 1446–1453 (2002).
44. Välimäki, V.-V. et al. Vitamin D Status as a Determinant of Peak Bone Mass in Young Finnish Men. J. Clin. Endocrinol. Metab. 89, 76–80 (2004).
45. Bolland, M. J. et al. Effect of calcium supplements on risk of myocardial infarction and cardiovascular events: Meta-analysis. BMJ 341, 289 (2010).
46. Revez, J. A. et al. Genome-wide association study identifies 143 loci associated with 25 hydroxyvitamin D concentration. Nat. Commun. 11, 1647 (2020).
47. Sarko, J. Bone and mineral metabolism. Emergency Medicine Clinics of North America vol. 23 703–721 (2005).
48. Wei, J., Hanna, T., Suda, N., Karsenty, G. & Ducy, P. Osteocalcin promotes β-cell proliferation during development and adulthood through Gprc6a. Diabetes 63, 1021–1031 (2014).
49. Oury, F. et al. XMaternal and offspring pools of osteocalcin influence brain development and functions. Cell 155, 228 (2013).
50. Bradburn, S. et al. Association between osteocalcin and cognitive performance in healthy older adults. Age Ageing 45, 844–849 (2016).
51. Oury, F. et al. Endocrine Regulation of Male Fertility by the Skeleton. Cell 144, 796–809 (2011).
52. Ducy, P. et al. Increased bone formation in osteocalcin-deficient mice. Nature 382, 448–452 (1996).
53. Huang, L., Yang, L., Luo, L., Wu, P. & Yan, S. Osteocalcin Improves Metabolic Profiles, Body Composition and Arterial Stiffening in an Induced Diabetic Rat Model. Exp. Clin. Endocrinol. Diabetes 125, 234–240 (2017).
54. Smith, C. et al. Osteocalcin and its forms across the lifespan in adult men. Bone 130, (2020).
55. Bonjour, J. P., Chevalley, T., Ferrari, S. & Rizzoli, R. The importance and relevance of peak bone mass in the prevalence of osteoporosis. Salud Publica Mex. 51, (2009).

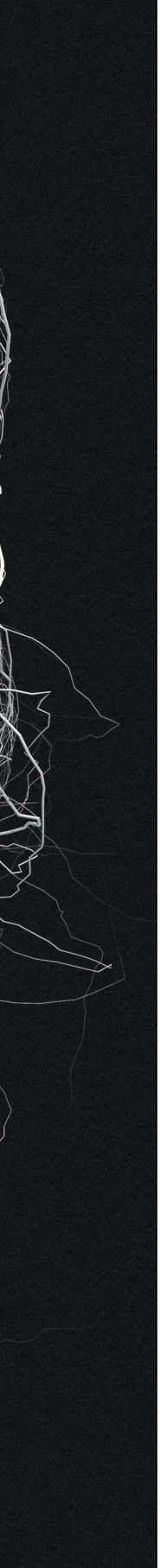
56. Hernandez, C. J., Beaupré, G. S. & Carter, D. R. A theoretical analysis of the relative influences of peak BMD, age-related bone loss and menopause on the development of osteoporosis. Osteoporos. Int. 14, 843–847 (2003).
57. Duan, Y., Wang, X. F., Evans, A. & Seeman, E. Structural and biomechanical basis of racial and sex differences in vertebral fragility in Chinese and Caucasians. Bone 36, 987–998 (2005).
58. Gordon, C. M. et al. The Determinants of Peak Bone Mass. Journal of Pediatrics vol. 180 261–269 (2017).
59. Mackey, D. C. et al. High-Trauma Fractures and Low Bone Mineral Density in Older Women and Men. JAMA 298, 2381 (2007).
60. Trajanoska, K. & Rivadeneira, F. The genetic architecture of osteoporosis and fracture risk. Bone 126, 2–10 (2019).
61. Trajanoska, K., Rivadeneira, F., Kiel, D. P. & Karasik, D. Genetics of Bone and Muscle Interactions in Humans. Curr. Osteoporos. Rep. 17, 86–95 (2019).
62. Zillikens, M. C. et al. Large meta-analysis of genome-wide association studies identifies five loci for lean body mass. Nat. Commun. 8, 80 (2017).
63. Fujimoto, K. et al. Use of bioelectrical impedance analysis for the measurement of appendicular skeletal muscle mass/whole fat mass and its relevance in assessing osteoporosis among patients with low back pain: A comparative analysis using Dual X-ray absorptiometry. Asian Spine J. 12, 839–845 (2018).
64. #1, Y.-F. P. et al. Genome-wide association study of appendicular lean mass in UK Biobank cohort 1. doi:10.1101/643536.
65. Willems, S. M. et al. Large-scale GWAS identifies multiple loci for hand grip strength providing biological insights into muscular fitness. 8, 16015 (2017).
66. Tikkanen, E. et al. Biological Insights Into Muscular Strength: Genetic Findings in the UK Biobank. Sci. Rep. 8, 6451 (2018).
67. Ho-Pham, L. T., Nguyen, U. D. T. & Nguyen, T. V. Association Between Lean Mass, Fat Mass, and Bone Mineral Density: A Meta-analysis. J. Clin. Endocrinol. Metab. 99, 30–38 (2014).
68. Medina-Gomez, C. et al. Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/TOM1L2 locus. Nat. Commun. 8, 121 (2017).
69. Chesmore, K., Bartlett, J. & Williams, S. M. The ubiquity of pleiotropy in human disease. Hum. Genet. 137, 39–44 (2018).
70. Pickrell, J. K. et al. Detection and interpretation of shared genetic influences on 42 human traits. Nat. Genet. 48, 709–717 (2016).
71. Yang, J., Lee, S. H., Goddard, M. E. & Visscher, P. M. GCTA: a tool for genome-wide complex trait analysis. Am. J. Hum. Genet. 88, 76–82 (2011).
72. Loh, P. R. et al. Contrasting genetic architectures of schizophrenia and other complex diseases using fast variance-components analysis. Nat. Genet. 47, 1385–1392 (2015).
73. Bulik-Sullivan, B. et al. An atlas of genetic correlations across human diseases and traits. Nat. Genet. 47, 1236–1241 (2015).
74. Huang, J., Johnson, A. D. & O'Donnell, C. J. PRIMe: A method for characterization and evaluation of pleiotropic regions from multiple genome-wide association studies. Bioinformatics 27, 1201–1206 (2011).

75. Cichonska, A. et al. metaCCA: summary statistics-based multivariate meta-analysis of genome-wide association studies using canonical correlation analysis. Bioinformatics 32, 1981–1989 (2016).
76. Casale, F. P., Rakitsch, B., Lippert, C. & Stegle, O. Efficient set tests for the genetic analysis of correlated traits. Nat. Methods 12, 755–758 (2015).
77. Cotsapas, C. et al. Pervasive sharing of genetic effects in autoimmune disease. PLoS Genet. 7, (2011).
78. Bhattacharjee, S. et al. A subset-based approach improves power and interpretation for the combined analysis of genetic association studies of heterogeneous traits. Am. J. Hum. Genet. 90, 821–835 (2012).
79. Vuckovic, D., Gasparini, P., Soranzo, N. & Iotchkova, V. MultiMeta: An R package for meta-analyzing multi-phenotype genome-wide association studies. Bioinformatics 31, 2754–2756 (2015).
80. Andreassen, O. A. et al. Improved Detection of Common Variants Associated with Schizophrenia and Bipolar Disorder Using Pleiotropy-Informed Conditional False Discovery Rate. PLoS Genet. 9, e1003455 (2013).
81. Turley, P. et al. Multi-trait analysis of genome-wide association summary statistics using MTAG. Nat. Genet. 50, 229–237 (2018).
82. Hackinger, S. & Zeggini, E. Statistical methods to detect pleiotropy in human complex traits. Open Biol. 7, 170125 (2017).
83. Yang, Q. & Wang, Y. Methods for analyzing multivariate phenotypes in genetic association studies. Journal of Probability and Statistics (2012) doi:10.1155/2012/652569.
84. Galesloot, T. E., van Steen, K., Kiemeney, L. A. L. M., Janss, L. L. & Vermeulen, S. H. A Comparison of Multivariate Genome-Wide Association Methods. PLoS One 9, e95923 (2014).
85. Watanabe, K. et al. A global overview of pleiotropy and genetic architecture in complex traits. Nat. Genet. 51, 1339–1348 (2019).
86. Bonifacio, E. et al. Genetic scores to stratify risk of developing multiple islet autoantibodies and type 1 diabetes: A prospective study in children. PLoS Med. 15, (2018).
87. Khera, A. V. et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. Nat. Genet. 50, 1219–1224 (2018).
88. Wünnemann, F. et al. Validation of Genome-Wide Polygenic Risk Scores for Coronary Artery Disease in French Canadians. Circ. Genomic Precis. Med. 12, e002481 (2019).
89. Halldorsdottir, T. et al. Polygenic risk: Predicting depression outcomes in clinical and epidemiological cohorts of youths. Am. J. Psychiatry 176, 615–625 (2019).
90. Estrada, K. et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat. Genet. 44, 491–501 (2012).
91. Forgetta, V. et al. Machine Learning to Predict Osteoporotic Fracture Risk from Genotypes. doi:10.1101/413716.
92. Khera, A. V. et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. Nat. Genet. 50, 1219–1224 (2018).
93. Elliott, L. T. et al. Genome-wide association studies of brain imaging phenotypes in UK Biobank. Nature 562, 210–216 (2018).
94. Zeggini, E., Gloyn, A. L., Barton, A. C. & Wain, L. V. Translational genomics and precision medicine: Moving from the lab to the clinic. Science (80-.). 365, 1409–1413 (2019).

95. Minion, J. T., Butcher, F., Timpson, N. & Murtagh, M. J. The ethics conundrum in Recall by Genotype (RbG) research: Perspectives from birth cohort participants. PLoS One 13, e0202502 (2018).
96. Finan, C. et al. The druggable genome and support for target identification and validation in drug development. Sci. Transl. Med. 9, (2017).
97. Nelson, M. R. et al. The support of human genetic evidence for approved drug indications. Nat. Genet. 47, 856–860 (2015).
98. Pushpakom, S. et al. Drug repurposing: Progress, challenges and recommendations. Nature Reviews Drug Discovery vol. 18 41–58 (2018).
99. Edwards, B. J., Haynes, C., Levenstien, M. A., Finch, S. J. & Gordon, D. Power and sample size calculations in the presence of phenotype errors for case/control genetic association studies. BMC Genet. 6, (2005).
100. Ji, F., Yang, Y., Haynes, C., Finch, S. J. & Gordon, D. Computing asymptotic power and sample size for case-control genetic association studies in the presence of phenotype and/or genotype misclassification errors. Stat. Appl. Genet. Mol. Biol. 4, (2006).
101. Manchia, M. et al. The Impact of Phenotypic and Genetic Heterogeneity on Results of Genome Wide Association Studies of Complex Diseases. PLoS One 8, e76295 (2013).
102. Dunham, I. et al. An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74 (2012).
103. Roadmap Epigenomics Consortium et al. Integrative analysis of 111 reference human epigenomes. Nature 518, 317–329 (2015).
104. Andersson, R. et al. An atlas of active enhancers across human cell types and tissues. Nature 507, 455–461 (2014).
105. Gallagher, M. D. & Chen-Plotkin, A. S. The Post-GWAS Era: From Association to Function. Am. J. Hum. Genet. 102, 717–730 (2018).
106. Morris, J. A. et al. An atlas of genetic influences on osteoporosis in humans and mice. Nat. Genet. 51, 258–266 (2019).
107. Deep learning vs machine learning: a simple way to understand the difference | Zendesk Blog. https://www.zendesk.com/blog/machine-learning-and-deep-learning/.
108. Hellwege, J. N. et al. Population Stratification in Genetic Association Studies. Curr. Protoc. Hum. Genet. 95, 1.22.1-1.22.23 (2017).
109. Martin, A. R. et al. Clinical use of current polygenic risk scores may exacerbate health disparities. Nat. Genet. 51, 584–591 (2019).
110. Duncan, L. et al. Analysis of polygenic risk score usage and performance in diverse human populations. Nat. Commun. 10, 3328 (2019).
111. Manrai, A. K. et al. Genetic Misdiagnoses and the Potential for Health Disparities. N. Engl. J. Med. 375, 655–665 (2016).
112. Gupta, M. et al. Identification of homogeneous genetic architecture of multiple genetically correlated traits by block clustering of genome-wide associations. J. Bone Miner. Res. 26, 1261–1271 (2011).
113. Gratten, J., Wray, N. R., Keller, M. C. & Visscher, P. M. Large-scale genomics unveils the genetic architecture of psychiatric disorders. Nature Neuroscience vol. 17 782–790 (2014).
114. Timpson, N. J., Greenwood, C. M. T., Soranzo, N., Lawson, D. J. & Richards, J. B. Genetic architecture: the shape of the genetic contribution to human traits and disease. Nat. Rev. Genet. 19, 110–124 (2018).

115. Ball, M. P. et al. Harvard Personal Genome Project: Lessons from participatory public research. Genome Med. 6, (2014).
116. Reuter, M. S. et al. The personal genome project Canada: Findings from whole genome sequences of the inaugural 56 participants. CMAJ 190, E126–E136 (2018).
117. Personal Genome Project UK (PGP-UK): a research and citizen science hybrid project in support of personalized medicine. BMC Med. Genomics 11, 108 (2018).
118. Munafò, M. R., Tilling, K., Taylor, A. E., Evans, D. M. & Davey Smith, G. Collider scope: when selection bias can substantially influence observed associations. Int. J. Epidemiol. 47, 226–235 (2018).
119. Pirastu, N. et al. Genetic analyses identify widespread sex-differential participation bias. bioRxiv 2020.03.22.001453 (2020) doi:10.1101/2020.03.22.001453.
120. Vassy, J. L. et al. The impact of whole-genome sequencing on the primary care and outcomes of healthy adult patients: A pilot randomized trial. Ann. Intern. Med. 167, 159–169 (2017).
121. Zheng, H. et al. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature 526, 112–7 (2015).
122. Styrkarsdottir, U. et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. Nature 497, 517–520 (2013).
123. Styrkarsdottir, U. et al. Two Rare Mutations in the COL1A2 Gene Associate with Low Bone Mineral Density and Fractures in Iceland. J. Bone Miner. Res. 31, 173–179 (2016).
124. Wainschtein, P. et al. Recovery of trait heritability from whole genome sequence data. bioRxiv 588020 (2019) doi:10.1101/588020.
125. Morris, J. A. et al. Epigenome-wide Association of DNA Methylation in Whole Blood With Bone Mineral Density. J. Bone Miner. Res. 32, 1644–1650 (2017).
126. Reppe, S. et al. Distinct DNA methylation profiles in bone and blood of osteoporotic and healthy postmenopausal women. Epigenetics 12, 674–687 (2017).
127. Sjögren, K. et al. The gut microbiota regulates bone mass in mice. J. Bone Miner. Res. 27, 1357–1367 (2012).
128. Nilsson, A. G., Sundh, D., Bäckhed, F. & Lorentzon, M. Lactobacillus reuteri reduces bone loss in older women with low bone mineral density: a randomized, placebo-controlled, double-blind, clinical trial. J. Intern. Med. 284, 307–317 (2018).





6

Summary

ENGLISH SUMMARY

As described in **Chapter 1** with the research embedded in this thesis, we aimed to provide new insights into the epidemiology of osteoporosis, sarcopenia and their sequels. We dedicated **Chapter 2** on the epidemiology of osteoporosis and fracture risk. In **Chapter 2.1** we focused on the observational studies on osteoporosis and fracture risk. In **Chapter 2.1.1** we showed that approximately 50% of all non-vertebral fractures occur above the osteoporosis threshold (T-score>-2.5) emphasizing the need to improve the screening of osteopenic patients. Furthermore, we presented that there is no difference in fracture trends between the periods of 1989–2001 and 2001–2013 in the Rotterdam Study. We also established that one single measurement of BMD to be a strong predictor of hip and other non-vertebral fractures over 20 years of follow-up. Next, in **Chapter 2.1.2** we observed that the effects of Metabolic syndrome (MS) on BMD are mainly driven by serum glucose levels; highlighting the importance of maintaining glycemic control in individuals with MS in order to pervert skeletal complications. **In Chapter 2.1.3** we showed a strong inverse association between total serum osteocalcin levels and BMD that was partly (~20%) explained by BMI. In addition, we highlighted that individuals with high serum osteocalcin levels and low BMI are more likely to be osteoporotic compared to individuals with osteocalcin and BMI levels within the normal ranges. In **Chapter 2.2** we focused on the genetic studies of osteoporosis and fracture risk. In **Chapter 2.2.1** we provided a general overview of the existing GWAS studies on osteoporotic traits and fracture risk. Briefly, more than ~90 loci have been found associated with DXA derived bone mineral density (BMD), over ~500 loci with heel estimated BMD and several others with other less widely available bone parameters such as bone geometry, shape, and microarchitecture. Notably, the majority of the known fracture loci are also BMD loci. In **Chapter 2.2.2** we demonstrated that total serum osteocalcin is complex heritable trait (h^2=9%). Part of the heritability was shared with type 2 diabetes; however, osteocalcin was not causally related with type 2 diabetes. In addition, we identified seven loci associated with total serum osteocalcin mapping to relevant bone pathways; highlighting the essential role of osteocalcin in bone biology. In **Chapter 2.2.3** we performed GWAS on TB-BMD, combining data across five age groups including children and older adults and identified 36 novel loci. We did not observe any large differences in the effects across age groups indicating that bone mass accrual is a key determinant of bone variability in the general population. In **Chapter 2.2.4** in a study of over 500,000 individuals (about 185,000 fracture cases), we provided evidence that the main genetic factors of fragility fractures also influence BMD, which was the only clinical risk factor to have shown an effect on fracture risk in community dwelling individuals. Most importantly, we showed that genetic predisposition to lower levels of vitamin D and estimated calcium intake

from dairy sources were not associated with fracture risk in our population. Our study fortifies the role of BMD as major risk factor of osteoporotic fracture and postulates that, among all the clinical risk factors we evaluated, interventions aimed at increasing BMD may have the biggest clinical impact. **In Chapter 2.2.5** we provided an overview of the Mendelian Randomization studies done in relation with bone outcomes. Till date MR studies only provide support for the causal effect of obesity on bone mineral density (BMD). Strikingly, studies have failed to prove a causal association between elevated vitamin D levels and higher BMD in community-dwelling individuals; which is in line with our findings on fracture risk. **Chapter 3** is focused on sarcopenia and its risk factors and consequences. In **Chapter 3.1,** we showed that muscle mass and power explain part of the variation in bone parameters as measured by pQCT and DXA. In addition, pQCT and DXA derived parameters correlated well; indicating that DXA may be sufficient in clinical practice. In **Chapter 3.2** we found that in the Rotterdam Study the prevalence of sarcopenia is relatively low but increased significantly with age, where 1 in 5 men and 1 in 10 women are sarcopenic by the age of 80 years. Lifestyle factors seem to play an essential role in the prevalence of sarcopenia. COPD was the only clinical risk factor that was more prevalent in individuals with presarcopenia and sarcopenia. The majority of individuals with sarcopenia have low BMD, suggesting that co-occurrence of both conditions may follow similar pathways and be responsive to similar interventions. In addition, our findings suggest that sarcopenia may not add additional risk on top of low BMD for fracture. In **Chapter 3.3** based on systematic **r**eview and meta-analysis of 10 studies (n=2,564 COPD patients) we estimated that the prevalence of sarcopenia in COPD patients ranged from 8% in population-based to 21% in clinic-based studies, and 63% in COPD patients residing in nursing homes. There was also large heterogeneity between studies stressing the need to develop standardized tests and cut-off points for sarcopenia that can be used in clinics. In **Chapter 3.4** we showed that lean mass muscle mass is associated with NAFLD but only in normal-weight women. Nevertheless, fat mass was a better predictor for NAFLD probability in both sexes. **In Chapter 4** we examined the pleiotropic effects in the musculoskeletal system**.** In **Chapter 4.1** we summarized the evidence from recent studies on the shared genetics between bone and muscle in humans. In **Chapter 4.2,** we identified 15 loci mapping in or near *MEF2C, XYLB/ACVR2B, PPP6R3/LRP5, LRP4* and *SLC27A6* among others, which were associated with both lean and bone mass. Our findings provide basis for future functional assessments aimed at unravelling biological mechanisms underlying complex bone-muscle interactions; with the potential of pinpointing strategies for the joint prevention and intervention of osteoporosis and sarcopenia. In **Chapter 4.3** we identified three loci (5q21.2, 7p21.3 and 19q12) associated with increased risk of falling. In addition, falls shared a proportion of their genetic variation with fracture risk, muscle strength, medication use and other risk

factors suggests potential pleiotropic relationships and common biological pathways could mediate diverse aspects of falling risk. The cerebellum tissue enrichment of falls-associated variants supports the mediation of postural balance in the etiology of falls. Our study provides novel biological insight that can be used for optimizing strategies directed at preventing falls and their associated deleterious consequences in aging individuals.In **Chapter 4.4** we performed systematic overlap analysis of OA and BMD using summary statistics from the GEFOS consortium and from the arcOGEN consortium and identified 143 variation with evidence for cross-phenotype association. Using this approach, we also identified a novel locus associated with OA mapping to the SMAD3 gene, relevant for bone remodelling and cartilage maintenance. Finally, in **Chapter 5** we discuss the implications of our findings and focus on the current gaps and limitations in the field that need to be overcome in future studies.

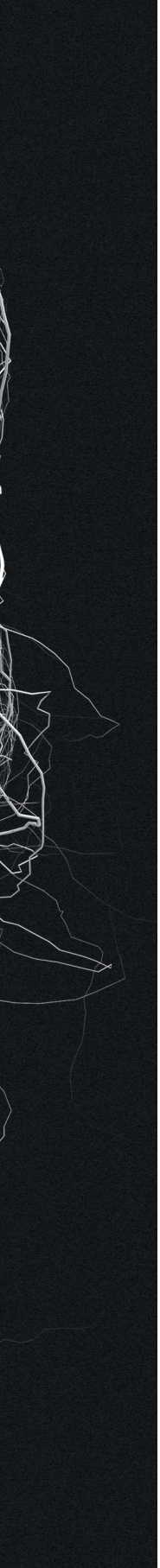
DUTCH SUMMARY

Zoals beschreven in **hoofdstuk 1** met het onderzoek dat in dit proefschrift is opgenomen, wilden we nieuwe inzichten verschaffen in de epidemiologie van osteoporose, sarcopenie en hun gevolgen. We hebben **hoofdstuk 2** gewijd aan de epidemiologie van osteoporose en fractuurrisico. In **hoofdstuk 2.1** hebben we ons gericht op de observationele studies naar osteoporose en fractuurrisico. In **hoofdstuk 2.1.1** hebben we aangetoond dat ongeveer 50% van alle niet-wervelfracturen optreden boven de osteoporosedrempel (T-score >-2,5), wat de noodzaak benadrukt om de screening van osteopenische patiënten te verbeteren. Verder hebben we in de Rotterdam Study laten zien dat er geen verschil is in breuktrends tussen de periodes 1989–2001 en 2001–2013. We hebben ook vastgesteld dat één enkele meting van BMD een sterke voorspeller is van heup- en andere niet-wervelfracturen gedurende 20 jaar follow-up. Vervolgens hebben we in **hoofdstuk 2.1.2** opgemerkt dat de effecten van het metabool syndroom (MS) op BMD voornamelijk worden aangedreven door serumglucosespiegels; het belang benadrukken van het handhaven van de glykemische controle bij personen met MS om skeletcomplicaties te voorkomen. In **hoofdstuk 2.1.3** toonden we een sterke inverse associatie tussen de totale serum osteocalcinespiegels en BMD die gedeeltelijk (~ 20%) werd verklaard door BMI. Daarnaast hebben we benadrukt dat personen met een hoge osteocalcinespiegel in het serum en een lage BMI meer kans hebben osteoporotisch te zijn dan personen met osteocalcine- en BMI-spiegels binnen het normale bereik. In **hoofdstuk 2.2** hebben we ons gericht op de genetische studies van osteoporose en fractuurrisico. In **hoofdstuk 2.2.1** hebben we een algemeen overzicht gegeven van de bestaande GWAS-onderzoeken naar osteoporotische eigenschappen en fractuurrisico. In het kort zijn meer dan ~ 90 loci gevonden geassocieerd met DXA-afgeleide botmineraaldichtheid (BMD), meer dan ~ 500 loci met door de hiel geschatte BMD en verschillende andere met andere minder algemeen beschikbare botparameters zoals botgeometrie, vorm en microarchitectuur. Met name de meerderheid van de bekende fractuur-loci zijn ook BMD-loci. In **hoofdstuk 2.2.2** hebben we aangetoond dat totaal osteocalcine in het serum een complexe erfelijke eigenschap is (h2 = 9%). Een deel van de erfelijkheidsgraad werd gedeeld met diabetes type 2; osteocalcine was echter niet causaal gerelateerd aan diabetes type 2. Daarnaast hebben we zeven loci geïdentificeerd die geassocieerd zijn met het in kaart brengen van osteocalcine in totaal serum naar relevante botroutes; het benadrukken van de essentiële rol van osteocalcine in de botbiologie. In **hoofdstuk 2.2.3** hebben we GWAS op TB-BMD uitgevoerd, waarbij we gegevens van vijf leeftijdsgroepen, waaronder kinderen en ouderen, hebben gecombineerd, en 36 nieuwe loci geïdentificeerd. We hebben geen grote verschillen waargenomen in de effecten tussen leeftijdsgroepen, wat erop wijst dat de opbouw van botmassa een belangrijke determinant

is van botvariabiliteit in de algemene populatie. In **hoofdstuk 2.2.4**, in een onderzoek onder meer dan 500.000 individuen (ongeveer 185.000 fractuurgevallen), hebben we bewijs geleverd dat de belangrijkste genetische factoren van breekbaarheidsfracturen ook BMD beïnvloeden, wat de enige klinische risicofactor was die een effect op fracturen vertoonde. risico bij thuiswonende individuen. Het belangrijkste was dat we aantoonden dat genetische aanleg voor lagere niveaus van vitamine D en geschatte calciuminname uit zuivelbronnen niet geassocieerd waren met het fractuurrisico in onze populatie. Onze studie versterkt de rol van BMD als een belangrijke risicofactor voor osteoporotische fracturen en veronderstelt dat van alle klinische risicofactoren die we hebben geëvalueerd, interventies gericht op het verhogen van BMD de grootste klinische impact kunnen hebben. In **Hoofdstuk 2.2.5** hebben we een overzicht gegeven van de Mendeliaanse Randomisatie studies uitgevoerd in relatie tot botuitkomsten. Tot op heden ondersteunen MR-onderzoeken alleen het causale effect van obesitas op de botmineraaldichtheid (BMD). Opvallend is dat studies geen oorzakelijk verband hebben aangetoond tussen verhoogde vitamine D-spiegels en hogere BMD bij thuiswonende individuen; wat in lijn is met onze bevindingen over fractuurrisico. **Hoofdstuk 3** is gericht op sarcopenie en de risicofactoren en gevolgen. In **hoofdstuk 3.1** hebben we laten zien dat spiermassa en spierkracht een deel van de variatie in botparameters verklaren, gemeten met pQCT en DXA. Bovendien correleerden de van pQCT en DXA afgeleide parameters goed; wat aangeeft dat DXA voldoende kan zijn in de klinische praktijk. In **hoofdstuk 3.2** ontdekten we dat in de Rotterdam Study de prevalentie van sarcopenie relatief laag is, maar significant toeneemt met de leeftijd, waar 1 op de 5 mannen en 1 op de 10 vrouwen sarcopenisch zijn op de leeftijd van 80 jaar. Leefstijlfactoren lijken een essentiële rol te spelen bij de prevalentie van sarcopenie. COPD was de enige klinische risicofactor die vaker voorkwam bij personen met presarcopenie en sarcopenie. De meerderheid van de personen met sarcopenie heeft een lage BMD, wat suggereert dat het gelijktijdig optreden van beide aandoeningen vergelijkbare routes kan volgen en reageert op vergelijkbare interventies. Bovendien suggereren onze bevindingen dat sarcopenie mogelijk geen extra risico toevoegt bovenop een lage BMD voor fracturen. In **hoofdstuk 3.3**, gebaseerd op systematische review en meta-analyse van 10 studies (n = 2.564 COPD-patiënten), schatten we dat de prevalentie van sarcopenie bij COPD-patiënten varieerde van 8% in de populatie tot 21% in klinisch gebaseerde studies, en 63 % bij COPD-patiënten die in verpleeghuizen wonen. Er was ook een grote heterogeniteit tussen studies die de noodzaak benadrukten om gestandaardiseerde tests en afkappunten voor sarcopenie te ontwikkelen die in klinieken kunnen worden gebruikt. In **hoofdstuk 3.4** hebben we laten zien dat spiermassa zonder spiermassa geassocieerd is met NAFLD, maar alleen bij vrouwen met een normaal gewicht. Desalniettemin was de vetmassa een betere voorspeller voor de NAFLD-waarschijnlijkheid bij beide geslachten. In **Hoofdstuk 4**

hebben we de pleiotrope effecten in het bewegingsapparaat onderzocht. In **hoofdstuk 4.1** vatten we het bewijs samen van recente studies over de gedeelde genetica tussen bot en spieren bij mensen. In **hoofdstuk 4.2** identificeerden we 15 loci-mapping in of nabij MEF2C, XYLB / ACvr2B, PPP6R3 / LRP5, LRP4 en SLC27A6, die geassocieerd waren met zowel magere als botmassa. Onze bevindingen vormen de basis voor toekomstige functionele beoordelingen gericht op het ontrafelen van biologische mechanismen die ten grondslag liggen aan complexe bot-spierinteracties; met het potentieel van aanwijsstrategieën voor de gezamenlijke preventie en interventie van osteoporose en sarcopenie. In **hoofdstuk 4.3** identificeerden we drie loci (5q21.2, 7p21.3 en 19q12) die geassocieerd zijn met een verhoogd valrisico. Bovendien deelden valpartijen een deel van hun genetische variatie met fractuurrisico, spierkracht, medicatiegebruik en andere risicofactoren, wat suggereert dat potentiële pleiotrope relaties en gemeenschappelijke biologische routes verschillende aspecten van het valrisico kunnen mediëren. De cerebellum weefselverrijking van valgerelateerde varianten ondersteunt de mediatie van het houdingsevenwicht in de etiologie van vallen. Onze studie biedt nieuw biologisch inzicht dat kan worden gebruikt voor het optimaliseren van strategieën die gericht zijn op het voorkomen van vallen en de bijbehorende schadelijke gevolgen bij ouder wordende individuen.In **hoofdstuk 4.4** hebben we systematische overlappende analyse van OA en BMD uitgevoerd met behulp van samenvattende statistieken van het GEFOS-consortium en van het arcOGEN-consortium en identificeerde 143 variaties met bewijs voor een cross-fenotype-associatie. Met behulp van deze aanpak identificeerden we ook een nieuwe locus geassocieerd met OA-mapping naar het SMAD3-gen, relevant voor botremodellering en kraakbeenonderhoud. Ten slotte bespreken we in **hoofdstuk 5** de implicaties van onze bevindingen en richten we ons op de huidige hiaten en beperkingen in het veld die in toekomstige studies moeten worden overwonnen.





7

Appendices

LIST OF PUBLICATIONS

Publications based on the studies described in this thesis

* Denotes equal contribution

** Denotes equal suprevision

Chapter 2.1.1

Trajanoska K, Schoufour JD, de Jonge EAL, Kieboom BCT, Mulder M, Stricker BH, Voortman T, Uitterlinden AG, Oei EHG, Ikram MA, Zillikens MC, Rivadeneira F, Oei L. Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. **Bone.** 2018 Sep; 114:116-124. doi: 10.1016/j.bone.2018.06.004.

Chapter 2.1.2

Trajanoska K, Medina-Gomez C, Zillikens MC, Uitterlinden AG, Rivadeneira F. The Association between Osteocalcin, Adiposity and Bone health: Results from the Rotterdam Study (**in preparation**)

Chapter 2.1.3

Muka T, **Trajanoska K,** Kiefte-de Jong JC, Oei L, Uitterlinden AG, Hofman A, Dehghan A, Zillikens MC, Franco OH, Rivadeneira F. The Association between Metabolic Syndrome, Bone Mineral Density, Hip Bone Geometry and Fracture Risk: The Rotterdam Study. **PLoS One**. 2015 Jun 12;10(6):e0129116. doi: 10.1371/journal.pone.0129116. eCollection 2015.

Chapter 2.2.1

Trajanoska K, Rivadeneira F. The genetic architecture of osteoporosis and fracture risk. **Bone**. 2019 Sep; 126:2-10. doi: 10.1016/j.bone.2019.04.005. Review.

Chapter 2.1.2

Trajanoska K*, Teumer A*, Karasik D, Uitterlinden AG , Rivadeneira F,. Kiel DP, Hsu YH on behalf of CHARGE and GEFOS consortia. Genome-wide association meta-analysis identifies six loci for osteocalcin levels (**in preparation**)

Chapter 2.1.3

Medina-Gomez C*, Kemp JP*, **Trajanoska K**, Luan J, Chesi A, Ahluwalia TS, Mook-Kanamori DO, Ham A, Hartwig FP, Evans DS, Joro R, Nedeljkovic I, Zheng HF, Zhu K, Atalay M, Liu CT, Nethander M, Broer L, Porleifsson G, Mullin BH, Handelman SK, Nalls MA, Jessen LE, Heppe DHM, Richards JB, Wang C, Chawes B, Schraut KE, Amin N, Wareham N, Karasik D, Van der Velde N, Ikram MA, Zemel BS, Zhou Y, Carlsson CJ, Liu Y, McGuigan FE,

Boer CG, Bønnelykke K, Ralston SH, Robbins JA, Walsh JP, Zillikens MC, Langenberg C, Li-Gao R, Williams FMK, Harris TB, Akesson K, Jackson RD, Sigurdsson G, den Heijer M, van der Eerden BCJ, van de Peppel J, Spector TD, Pennell C, Horta BL, Felix JF, Zhao JH, Wilson SG, de Mutsert R, Bisgaard H, Styrkársdóttir U, Jaddoe VW, Orwoll E, Lakka TA, Scott R, Grant SFA, Lorentzon M, van Duijn CM, Wilson JF, Stefansson K, Psaty BM, Kiel DP, Ohlsson C, Ntzani E, van Wijnen AJ, Forgetta V, Ghanbari M, Logan JG, Williams GR, Bassett JHD, Croucher PI, Evangelou E, Uitterlinden AG, Ackert-Bicknell CL, Tobias JH, Evans DM, Rivadeneira F. Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects. **Am J Hum Genet.** 2018 Jan 4;102(1):88-102. doi: 10.1016/j.ajhg.2017.12.005.

Chapter 2.1.4

Trajanoska K*, Morris JA*, Oei L*, Zheng HF*, Evans DM**, Kiel DP**, Ohlsson C**, Richards JB**, Rivadeneira F**; GEFOS/GENOMOS consortium and the 23andMe research team. Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study. **BMJ.** 2018 Aug 29;362:k3225. doi: 10.1136/bmj.k3225.

Chapter 2.1.5

Trajanoska K, Rivadeneira F. Using Mendelian Randomization to Decipher Mechanisms of Bone Disease. **Curr Osteoporos Rep**. 2018 Oct;16(5):531-540. doi: 10.1007/s11914-018-0467-3. Review.

Chapter 3.1

Witlox G, Voortman T, Rivadeneira F, **Trajanoska K.** Association between muscle force and power with different pQCT and DXA-derived parameters in middle-aged adults (**in preparation**)

Chapter 3.2

Trajanoska K, Schoufour JD, Darweesh SK, Benz E, Medina-Gomez C, Alferink LJ, Lahousse L, Brusselle G, Stricker B, Darwish Murad S, Zillikens MC, Uitterlinden AG, Ikram MA, Franco OH, Rivadeneira F. Sarcopenia and Its Clinical Correlates in the General Population: The Rotterdam Study. **J Bone Miner Res**. 2018 Jul;33(7):1209-1218. doi: 10.1002/jbmr.3416. Epub 2018 Apr 17.

Chapter 3.3

Benz E*,**Trajanoska K***, Lahousse L, Schoufour JD, Terzikhan N, De Roos E, de Jonge GB, Williams R, Franco OH, Brusselle G, Rivadeneira F. Sarcopenia in COPD: a systematic

review and meta-analysis **Eur Respir Rev.** 2019 Nov 13;28(154). pii: 190049. doi: 10.1183/16000617.0049-2019. Print 2019 Dec 31. Review.

Chapter 3.4

Alferink LJM,**Trajanoska K**, Erler NS, Schoufour JD, de Knegt RJ, Ikram MA, Janssen HLA, Franco OH, Metselaar HJ, Rivadeneira F, Darwish Murad S. J Nonalcoholic Fatty Liver Disease in The Rotterdam Study: About Muscle Mass, Sarcopenia, Fat Mass, and Fat Distribution. **J Bone Miner Res**. 2019 Jul;34(7):1254-1263. doi: 10.1002/jbmr.3713. Epub 2019 May 10.

Chapter 4.1

Trajanoska K, Rivadeneira F, Kiel DP, Karasik D.Genetics of Bone and Muscle Interactions in Humans. **Curr Osteoporos Rep**. 2019 Apr;17(2):86-95. doi: 10.1007/s11914-019-00505-1. Review.

Chapter 4.2

Trajanoska K, Dimou NL, Karasik D, Rivadeneira F on behalf of GEFOS consortium
Leveraging data from bivariate genome-wide association meta-analysis to unravel novel pleitropic mechanisms of bone-muscle crosstalk (**in preparation**)

Chapter 4.3

Trajanoska K, Lotta SJ, Medina-Gomez C, Hsu YH, Zhou S, van Schoor NM, de Groot C.P.G.M. L, Karasik D, Richards JB, DouglasKP, Uitterlinden AG, Perry JRB, van der Velde N, Day FR **, Rivadeneira F**. Genetic basis of falling risk susceptibility in the UK Biobank Study. Communications Biology (**in press**)

Chapter 4.4

Hackinger S, **Trajanoska K**, Styrkarsdottir U, Zengini E, Steinberg J, Ritchie GRS, Hatzikotoulas K, Gilly A, Evangelou E, Kemp JP; arcOGEN Consortium, GEFOS Consortium, Evans D, Ingvarsson T, Jonsson H, Thorsteinsdottir U, Stefansson K, McCaskie AW, Brooks RA, Wilkinson JM, Rivadeneira F, Zeggini E. Evaluation of shared genetic aetiology between osteoarthritis and bone mineral density identifies SMAD3 as a novel osteoarthritis risk locus. **Hum Mol Genet**. 2017 Oct 1;26(19):3850-3858. doi: 10.1093/hmg/ddx285

Other publications

1. Waqas K, Chen J, Koromani F, **Trajanoska K**, van der Eerden BC, Uitterlinden AG, Rivadeneira F, Zillikens MC. "*Skin Autofluorescence, a Noninvasive Biomarker for Advanced Glycation End-Products, Is Associated with Prevalent Vertebral and Major Osteoporotic Fractures: The Rotterdam Study*" **J Bone Miner Res.** 2020 May 28. doi: 10.1002/jbmr.4096. Online ahead of print.
2. Magnus MC, Guyatt AL, Lawn RB, Wyss AB, **Trajanoska K,** Küpers LK, Rivadeneira F, Tobin MD, London SJ, Lawlor DA, Millard LAC, Fraser A. "*Identifying potential causal effects of age at menarche: a Mendelian randomization phenome-wide association study*". **BMC Med**. 2020 Mar 23;18(1):71. doi: 10.1186/s12916-020-01515-y.
3. Oliai Araghi S, Kiefte-de Jong JC, **Trajanoska K**, Koromani F, Rivadeneira F, Zillikens MC, van Schoor NM, de Groot LCPGM, Ikram MA, Uitterlinden AG, Stricker BH, van der Velde N. "*Do Vitamin D Level and Dietary Calcium Intake Modify the Association Between Loop Diuretics and Bone Health?*" **Calcif Tissue Int**. 2020 Feb;106(2):104-114. doi: 10.1007/s00223-019-00621-1.
4. Koromani F, Oei L, Shevroja E, **Trajanoska K**, Schoufour J, Muka T, Franco OH, Ikram MA, Zillikens MC, Uitterlinden AG, Krestin GP, Anastassiades T, Josse R, Kaiser SM, Goltzman D, Lentle BC, Prior JC, Leslie WD, McCloskey E, Lamy O, Hans D, Oei EH, Rivadeneira F. "*Vertebral Fractures in Individuals With Type 2 Diabetes: More Than Skeletal Complications Alone.*" **Diabetes Care**. 2020 Jan;43(1):137-144. doi: 10.2337/dc19-0925.
5. Cerani A, Zhou S, Forgetta V, Morris JA, **Trajanoska K**, Rivadeneira F, Larsson SC, Michaëlsson K, Richards JB. Genetic predisposition to increased serum calcium, bone mineral density, and fracture risk in individuals with normal calcium levels: mendelian randomisation study.**BMJ**. 2019 Aug 1;366:l4410. doi: 10.1136/bmj.l4410.
6. Hsu YH, Estrada K, Evangelou E, Ackert-Bicknell C, Akesson K, Beck T, Brown SJ, Capellini T, Carbone L, Cauley J, Cheung CL, Cummings SR, Czerwinski S, Demissie S, Econs M, Evans D, Farber C, Gautvik K, Harris T, Kammerer C, Kemp J, Koller DL, Kung A, Lawlor D, Lee M, Lorentzon M, McGuigan F, Medina-Gomez C, Mitchell B, Newman A, Nielson C, Ohlsson C, Peacock M, Reppe S, Richards JB, Robbins J, Sigurdsson G, Spector TD, Stefansson K, Streeten E, Styrkarsdottir U, Tobias J, **Trajanoska K**, Uitterlinden A, Vandenput L, Wilson SG, Yerges-Armstrong L, Young M, Zillikens MC, Rivadeneira F, Kiel DP, Karasik D. "*Meta-Analysis of Genomewide Association Studies Reveals Genetic Variants for Hip Bone Geometry.*" **J Bone Miner Res**. 2019 Jul;34(7):1284-1296. doi: 10.1002/jbmr.3698.
7. Koromani F, **Trajanoska K**, Rivadeneira F, Oei L. "*Recent Advances in the Genetics of Fractures in Osteoporosis.*" **Front Endocrinol** (Lausanne). 2019 Jun 4;10:337. doi: 10.3389/fendo.2019.00337. eCollection 2019. Review.
8. Styrkarsdottir U, Stefansson OA, Gunnarsdottir K, Thorleifsson G, Lund SH, Stefansdottir L, Juliusson K, Agustsdottir AB, Zink F, Halldorsson GH, Ivarsdottir EV, Benonisdottir S, Jonsson H, Gylfason A, Norland K,**Trajanoska K**, Boer CG, Southam L, Leung JCS, Tang NLS, Kwok TCY, Lee JSW, Ho SC, Byrjalsen I, Center JR, Lee SH, Koh JM, Lohmander LS, Ho-Pham LT, Nguyen TV, Eisman JA, Woo J, Leung PC, Loughlin J, Zeggini E, Christiansen C, Rivadeneira F, van Meurs J, Uitterlinden AG, Mogensen B, Jonsson H, Ingvarsson T, Sigurdsson G, Benediktsson R, Sulem P, Jonsdottir I, Masson G, Holm H, Norddahl GL, Thorsteinsdottir U, Gudbjartsson DF, Stefansson K. "*GWAS of bone size yields twelve loci that also affect height,*

BMD, osteoarthritis or fractures." **Nat Commun**. 2019 May 3;10(1):2054. doi: 10.1038/s41467-019-09860-0. Erratum in:Nat Commun. 2019 May 24;10(1):2358.

9. Grgic O, Rivadeneira F, Shevroja E, **Trajanoska K**, Jaddoe VWV, Uitterlinden AG, Beck TJ, Wolvius EB, Medina-Gomez C. "*Femoral stress is prominently associated with fracture risk in children: The Generation R Study.*" **Bone**. 2019 May;122:150-155. doi: 10.1016/j.bone.2019.02.018.
10. Grgic O, Chung K, Shevroja E, **Trajanoska K**, Uitterlinden AG, Wolvius EB, Rivadeneira F, Medina-Gomez C. "*Fractures in school age children in relation to sex and ethnic background: The Generation R Study."* **Bone**. 2019 Apr;121:227-231. doi: 10.1016/j.bone.2019.01.019.
11. Schoufour JD, Franco OH, Kiefte-de Jong JC, **Trajanoska K**, Stricker B, Brusselle G, Rivadeneira F, Lahousse L, Voortman T. The association between dietary protein intake, energy intake and physical frailty: results from the Rotterdam Study. **Br J Nutr**. 2019 Feb;121(4):393-401. doi: 10.1017/S0007114518003367.
12. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, Vulpescu NA, Forgetta V, Kleinman A, Mohanty ST, Sergio CM, Quinn J, Nguyen-Yamamoto L, Luco AL, Vijay J, Simon MM, Pramatarova A, Medina-Gomez C, **Trajanoska K**, Ghirardello EJ, Butterfield NC, Curry KF, Leitch VD, Sparkes PC, Adoum AT, Mannan NS, Komla-Ebri DSK, Pollard AS, Dewhurst HF, Hassall TAD, Beltejar MG; 23andMe Research Team, Adams DJ, Vaillancourt SM, Kaptoge S, Baldock P, Cooper C, Reeve J, Ntzani EE, Evangelou E, Ohlsson C, Karasik D, Rivadeneira F, Kiel DP, Tobias JH, Gregson CL, Harvey NC, Grundberg E, Goltzman D, Adams DJ, Lelliott CJ, Hinds DA, Ackert-Bicknell CL, Hsu YH, Maurano MT, Croucher PI, Williams GR, Bassett JHD, Evans DM, Richards JB. "*An atlas of genetic influences on osteoporosis in humans and mice."* **Nat Genet.** 2019 Feb;51(2):258-266. doi: 10.1038/s41588-018-0302-x. Epub 2018 Dec 31. Erratum in:Nat Genet. 2019 May;51(5):920.
13. Rivadeneira F, **Trajanoska K**, Morris J, Richards JB. "*Authors' reply to Sugiyama."* **BMJ**. 2019 Jan 14;364:l115. doi: 10.1136/bmj.l115.
14. Veldscholte K*, Barjaktarovic M*, **Trajanoska K**, Jaddoe VWV, Visser TJ, de Rijke YB, Peeters RP, Rivadeneira F, Korevaar TIM. "*The Association of Thyroid Function With Bone Density During Childhood."* **J Clin Endocrinol Metab**. 2018 Nov 1;103(11):4125-4134. doi: 10.1210/jc.2018-00294.
15. Warrington NM, Shevroja E, Hemani G, Hysi PG, Jiang Y, Auton A, Boer CG, Mangino M, Wang CA, Kemp JP, McMahon G, Medina-Gomez C, Hickey M, **Trajanoska K**, Wolke D, Ikram MA; 23andMe Research Team, Montgomery GW, Felix JF, Wright MJ, Mackey DA, Jaddoe VW, Martin NG, Tung JY, Davey Smith G, Pennell CE, Spector TD, van Meurs J, Rivadeneira F, Medland SE, Evans DM. "*Genome-wide association study identifies nine novel loci for 2D:4D finger ratio, a putative retrospective biomarker of testosterone exposure in utero."* **Hum Mol Genet**. 2018 Jun 1;27(11):2025-2038. doi: 10.1093/hmg/ddy121.
16. Kemp JP*, Morris JA*, Medina-Gomez C, Forgetta V, Warrington NM, Youlten SE, Zheng J, Gregson CL, Grundberg E, **Trajanoska K**, Logan JG, Pollard AS, Sparkes PC, Ghirardello EJ, Allen R, Leitch VD, Butterfield NC, Komla-Ebri D, Adoum AT, Curry KF, White JK, Kussy F, Greenlaw KM, Xu C, Harvey NC, Cooper C, Adams DJ, Greenwood CMT, Maurano MT, Kaptoge S, Rivadeneira F, Tobias JH, Croucher PI, Ackert-Bicknell CL, Bassett JHD, Williams GR, Richards JB, Evans DM. "*Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis."* **Nat Genet**. 2017 Oct;49(10):1468-1475. doi: 10.1038/ng.3949.

17. Morris JA*, Tsai PC*, Joehanes R*, Zheng J*, **Trajanoska K*,** Soerensen M*, Forgetta V, Castillo-Fernandez JE, Frost M, Spector TD, Christensen K, Christiansen L, Rivadeneira F, Tobias JH, Evans DM, Kiel DP, Hsu YH, Richards JB, Bell JT. "*Epigenome-wide Association of DNA Methylation in Whole Blood With Bone Mineral Density.*" **J Bone Miner Res**. 2017 Aug;32(8):1644-1650. doi: 10.1002/jbmr.3148.
18. Willems SM*, Wright DJ*, Day FR, **Trajanoska K**, Joshi PK, Morris JA, Matteini AM, Garton FC, Grarup N, Oskolkov N, Thalamuthu A, Mangino M, Liu J, Demirkan A, Lek M, Xu L, Wang G, Oldmeadow C, Gaulton KJ, Lotta LA, Miyamoto-Mikami E, Rivas MA, White T, Loh PR, Aadahl M, Amin N, Attia JR, Austin K, Benyamin B, Brage S, Cheng YC, Cięszczyk P, Derave W, Eriksson KF, Eynon N, Linneberg A, Lucia A, Massidda M, Mitchell BD, Miyachi M, Murakami H, Padmanabhan S, Pandey A, Papadimitriou I, Rajpal DK, Sale C, Schnurr TM, Sessa F, Shrine N, Tobin MD, Varley I, Wain LV, Wray NR, Lindgren CM, MacArthur DG, Waterworth DM, McCarthy MI, Pedersen O, Khaw KT, Kiel DP; GEFOS Any-Type of Fracture Consortium, Pitsiladis Y, Fuku N, Franks PW, North KN, van Duijn CM, Mather KA, Hansen T, Hansson O, Spector T, Murabito JM, Richards JB, Rivadeneira F, Langenberg C, Perry JRB, Wareham NJ, Scott RA. "*Large-scale GWAS identifies multiple loci for hand grip strength providing biological insights into muscular fitness.*" **Nat Commun**. 2017 Jul 12;8:16015. doi: 10.1038/ncomms16015.
19. Tachmazidou I, Süveges D, Min JL, Ritchie GRS, Steinberg J, Walter K, Iotchkova V, Schwartzentruber J, Huang J, Memari Y, McCarthy S, Crawford AA, Bombieri C, Cocca M, Farmaki AE, Gaunt TR, Jousilahti P, Kooijman MN, Lehne B, Malerba G, Männistö S, Matchan A, Medina-Gomez C, Metrustry SJ, Nag A, Ntalla I, Paternoster L, Rayner NW, Sala C, Scott WR, Shihab HA, Southam L, St Pourcain B, Traglia M, **Trajanoska K**, Zaza G, Zhang W, Artigas MS, Bansal N, Benn M, Chen Z, Danecek P, Lin WY, Locke A, Luan J, Manning AK, Mulas A, Sidore C, Tybjaerg-Hansen A, Varbo A, Zoledziewska M, Finan C, Hatzikotoulas K, Hendricks AE, Kemp JP, Moayyeri A, Panoutsopoulou K, Szpak M, Wilson SG, Boehnke M, Cucca F, Di Angelantonio E, Langenberg C, Lindgren C, McCarthy MI, Morris AP, Nordestgaard BG, Scott RA, Tobin MD, Wareham NJ; SpiroMeta Consortium; GoT2D Consortium, Burton P, Chambers JC, Smith GD, Dedoussis G, Felix JF, Franco OH, Gambaro G, Gasparini P, Hammond CJ, Hofman A, Jaddoe VWV, Kleber M, Kooner JS, Perola M, Relton C, Ring SM, Rivadeneira F, Salomaa V, Spector TD, Stegle O, Toniolo D, Uitterlinden AG; arcOGEN Consortium; Understanding Society Scientific Group; UK10K Consortium, Barroso I, Greenwood CMT, Perry JRB, Walker BR, Butterworth AS, Xue Y, Durbin R, Small KS, Soranzo N, Timpson NJ, Zeggini E. "*Whole-Genome Sequencing Coupled to Imputation Discovers Genetic Signals for Anthropometric Traits.*" **Am J Hum Genet**. 2017 Jun 1;100(6):865-884. doi: 10.1016/j.ajhg.2017.04.014.
20. Medina-Gomez C, Heppe DHM, Yin JL, **Trajanoska K**, Uitterlinden AG, Beck TJ, Jaddoe VWV, Rivadeneira F. "*Bone Mass and Strength in School-Age Children Exhibit Sexual Dimorphism Related to Differences in Lean Mass: The Generation R Study.*" **J Bone Miner Res**. 2016 May;31(5):1099-106. doi: 10.1002/jbmr.2755.
21. Zheng HF, Forgetta V, Hsu YH, Estrada K, Rosello-Diez A, Leo PJ, Dahia CL, Park-Min KH, Tobias JH, Kooperberg C, Kleinman A, Styrkarsdottir U, Liu CT, Uggla C, Evans DS, Nielson CM, Walter K, Pettersson-Kymmer U, McCarthy S, Eriksson J, Kwan T, Jhamai M, **Trajanoska K**, Memari Y, Min J, Huang J, Danecek P, Wilmot B, Li R, Chou WC, Mokry LE, Moayyeri A, Claussnitzer M, Cheng CH, Cheung W, Medina-Gómez C, Ge B, Chen SH, Choi K, Oei L, Fraser J, Kraaij R, Hibbs MA, Gregson CL, Paquette D, Hofman A, Wibom C, Tranah GJ, Marshall M, Gardiner BB, Cremin K, Auer P, Hsu L, Ring S, Tung JY, Thorleifsson G, Enneman AW,

van Schoor NM, de Groot LC, van der Velde N, Melin B, Kemp JP, Christiansen C, Sayers A, Zhou Y, Calderari S, van Rooij J, Carlson C, Peters U, Berlivet S, Dostie J, Uitterlinden AG, Williams SR, Farber C, Grinberg D, LaCroix AZ, Haessler J, Chasman DI, Giulianini F, Rose LM, Ridker PM, Eisman JA, Nguyen TV, Center JR, Nogues X, Garcia-Giralt N, Launer LL, Gudnason V, Mellström D, Vandenput L, Amin N, van Duijn CM, Karlsson MK, Ljunggren Ö, Svensson O, Hallmans G, Rousseau F, Giroux S, Bussière J, Arp PP, Koromani F, Prince RL, Lewis JR, Langdahl BL, Hermann AP, Jensen JE, Kaptoge S, Khaw KT, Reeve J, Formosa MM, Xuereb-Anastasi A, Åkesson K, McGuigan FE, Garg G, Olmos JM, Zarrabeitia MT, Riancho JA, Ralston SH, Alonso N, Jiang X, Goltzman D, Pastinen T, Grundberg E, Gauguier D, Orwoll ES, Karasik D, Davey-Smith G; AOGC Consortium, Smith AV, Siggeirsdottir K, Harris TB, Zillikens MC, van Meurs JB, Thorsteinsdottir U, Maurano MT, Timpson NJ, Soranzo N, Durbin R, Wilson SG, Ntzani EE, Brown MA, Stefansson K, Hinds DA, Spector T, Cupples LA, Ohlsson C, Greenwood CM; UK10K Consortium, Jackson RD, Rowe DW, Loomis CA, Evans DM, Ackert-Bicknell CL, Joyner AL, Duncan EL, Kiel DP, Rivadeneira F, Richards JB. "*Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture.*" **Nature**. 2015 Oct 1;526(7571):112-7. doi: 10.1038/nature14878.

22. Garan Jones*, **Katerina Trajanoska*,** Adam J Santanasto*, Najada Stringa*, Chia-Ling Kuo*, Janice L Atkins*, Joshua R Lewis, ThuyVy Duong, Shengjun Hong, Mary L Biggs, Jian'an Luan, Chloe Sarnowski, Kathryn L Lunetta, Toshiko Tanaka, Mary K Wojczynski, Ryan Cvejkus, Maria Nethander, Sahar Ghasemi, Jingyun Yang, M. Carola Zillikens, Stefan Walter, Kamil Sicinski, Erika Kague, Cheryl L AckertBicknell, Dan E Arking, B Gwen Windham, Eric Boerwinkle, Megan L Grove, Misa Graff, Dominik Spira, Ilja Demuth, Nathalie van der Velde, Lisette C P G M de Groot, Bruce M Psaty, Michelle C Odden, Alison E Fohner, Claudia Langenberg, Nicholas J Wareham, Stefania Bandinelli, Natasja M van Schoor, Martijn Huisman, Qihua Tan, Joseph Zmuda, Dan Mellström, Magnus Karlsson, David A Bennett, Aron S Buchman, Philip L De Jager, Andre G Uitterlinden, Uwe Völker, Thomas Kocher, Alexander Teumer, Leocadio Rodriguéz-Mañas, Francisco J García García, José A Carnicero, Pamela Herd, Lars Bertram, Claes Ohlsson, Joanne M Murabito*, George A Kuchel*, Luigi Ferrucci*, David Melzer*, David Karasik*,Fernando Rivadeneira*, Douglas P Kiel*, Luke C Pilling* "*Genome-wide meta-analysis of muscle weakness identifies 15 susceptibility loci in older men and women*" (**submitted**)
23. van der Schaft N, **Trajanoska K**, Rivadeneira F, Ikram M A, Schoufour D J, Voortman T "*Total Dietary Antioxidant Capacity and Longitudinal Trajectories of Body Composition*" (**submitted**)
24. Bergink A, **Trajanoska K**, Uitterlinden A, van Meurs J. "*Mendelian Randomization study on vitamin D lelves and osteoarthritis risk: a concise report*" (**submitted**)
25. Benz E , **Trajanoska K**, Schoufour D J, Lahousse L, de Roos W E, Terzikhan N, Medina-Gomez C, Verhamme K, Williams R, Stricker H B, Franco H O, Ikram M A, Rivadeneira F, Brusselle G "*Sarcopenia in elderly population with chronic airway diseases: the Rotterdam study*" (**submitted**)

PHD PORTFOLIO SUMMARY

Name PhD student:	Katerina Trajanoska
PhD period:	August 2014 – August 2019
Erasmus MC Department:	Internal Medicine & Epidemiology
Promotor(s):	Prof. dr. A.G Uitterlinden and Prof. dr. F. Rivadeneira
Supervisor:	Dr. C. Medina-Gomez
Research School:	NIHES: Erasmus University

GENERAL RESEARCH SKILLS

	Year	ECTS
Master of science in Health Sciences (NIHES)	**2014-2015**	**70**
Specialization: Genetic Epidemiology		
o Principles of Research in Medicine		0.7
o Genome Wide Association Analysis		1.4
o Principals of Genetic Epidemiology		0.7
o Genomics in Molecular Medicine		1.4
o Social Epidemiology		0.7
o Advances in Genomic Research		0.4
o Logistic regression		1.4
o Introduction to Bayesian Methods in Clinical and Epidemiological Research		1.4
o Causal Mediation Analysis		0.7
o Study Design		4.3
o Biostatistical Methods I: Basic Principles		5.7
o Biostatistical Methods II: Classical Regression Models		4.3
o Genetic-Epidemiologic Research Methods		5.1
o SNP's and Human Diseases		1.4
o Linux for Scientists		0.6
Advanced courses		
o Repeated Measurements in Clinical Study		1.4
o Advances in Genome-Wide Association Studies		1.4
o Family-based Genetic Analysis		1.4
o A first encounter with next generation sequencing data		1.4
o Mendelian Randomization, Cambridge	2016	1.0
Skills Courses		
o Introduction to Medical writing		1.1
o Courses for the Quantitative Researcher		1.4
o Systematical literature retrieval in other databases	2018	0.6
o Molmed: Photoshop and Illustrator CC Workshop	2019	0.3

NATIONAL CONFERENCES AND PRESENTATIONS

	Year	ECTS
o Department of Internal Medicine, Endocrinology research general meetings	2014-2019	1.0
o Department of Internal Medicine, Laboratory of Human genetics meetings	2014-2019	1.0
o Molecular Epidemiology Research meetings	2014-2019	1.0
o Wetenschappendagen Internal Medicine Department	2015-2019	1.5
-Poster presentations		0.7
o Dutch Society of Calcium and bone	2014-2018	1.5
-Oral Presentations		1.2

INTERNATIONAL CONFERENCES AND PRESENTATIONS

o **European Calcified Tissue Society, Rome**	2016	1.5
-Poster presentation		0.5
o **American Society of Bone and Mineral Research, Atlanta**	2016	1.5
-Poster presentation		0.5
European Calcified Tissue Society, Salzburg	2017	1.5
-Oral poster presentation		0.7
-Oral presentation		0.7
o **International Conference on Frailty and Sarcopenia Research, Barcelona**	2017	1.5
o Oral presentation		0.7
o **CHARGE meeting, New York**	2017	1.0
-Poster presentation		0.5
o **European Calcified Tissue Society, Valencia**	2018	1.5
-Oral Poster presentation		0.7
o **American Society of Bone and Mineral Research, Montreal**	2018	1.5
-Plenary Poster		0.7
o **CHARGE meeting, Rotterdam**	2018	1.0
-Poster presentation		0.5
o **European Calcified Tissue Society, Budapest**	2019	1.5
-Oral Presentation		0.7
-Oral Poster presentation		0.7
-Poster presentation		0.5
o **American Society of Bone and Mineral Research, Orlando**	2019	1.5
-Plenary Poster		0.7

TEACHING ACTIVITIES

	Year	ECTS
o **Teaching assistant at NIHES Erasmus Summer Programme**		4.0
-Genomics in molecular medicine	2016-2019	
-Genome-wide association studies	2016-2019	
- Principles of Research in Medicine and Epidemiology	2018	
o **Teaching assistant at Molmed course**	2017-2019	0.7
-SNPs and Human Diseases practical's		
o **Teaching assistant at NIHES Master course**		
-Genetic-epidemiologic Research Methods	2019	1.0
-Biostatistical Methods I: Basic Principles	2017, 2019	1.0

OTHER

o Research project supervisor – 4th year medical students	2018-2019	2.0
o Master Student supervision	2019-present	1.0
-Artemis Gkitakou – sleep problems and musculoskeletal health		
o Peer review of articles for scientific journals	2017-2020	2.0
-Journal of Bone and Mineral Research, Osteoporosis International, Bone, Scientific Reports, Frontiers of Endocrinology, European Journal of Epidemiology		

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Frank Herbert

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Katerina Trajanoska was born on 7th August 1988 in Skopje, Macedonia. In 2007, she graduated as health nurse from the medical high school "Dr Pance Karagjozov" in Skopje, Macedonia. The same year she enrolled at the "Ss. Cyril and Methodius University" in Skopje where she obtained her Medical Doctor's degree in 2013 and a year later successfully passed the medical board exam. During medical school, in 2012, she was granted the ERAWEB Scholarship and went to the University of Rotterdam in the Netherlands for the first time, to perform 4-month training in Clinical Epidemiology. In 2014 she returns to Erasmus Medical Center as a PhD exchange student under ERAWEB scholarship. In 2015, she received her NIHES MSc in Genetic Epidemiology at Erasmus MC and expanded her research project in her current PhD-project entitled "The interplay of bone and muscle in health and disease" under the supervision of Prof. dr. Andre G Uitterlinden, Prof. dr. Fernando Rivadeneira and Dr. Carolina Medina-Gomez. She worked on musculoskeletal health looking at the associations between environmental and genetic factors and various musculoskeletal outcomes.