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.

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