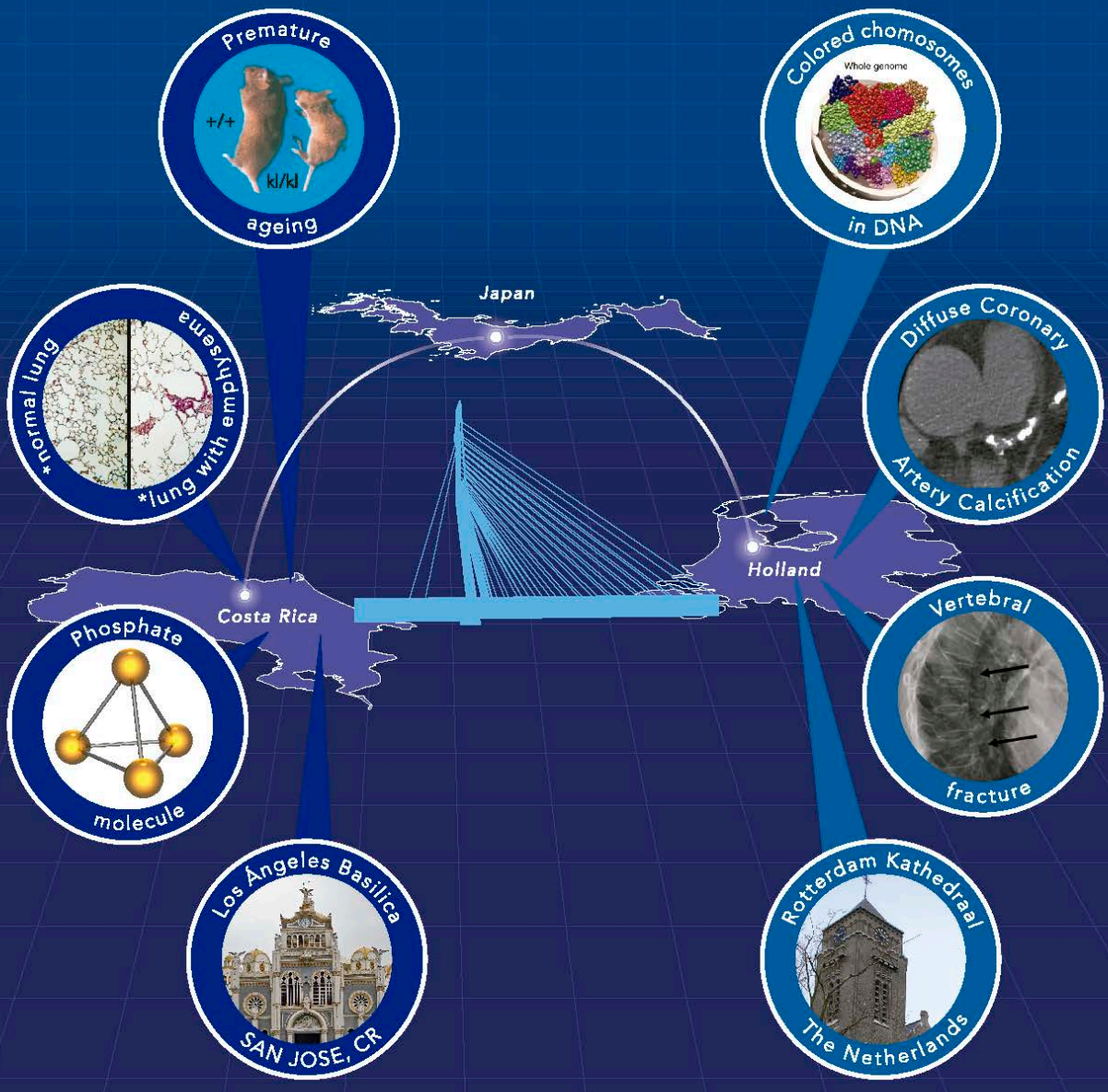
BONE & PHOSPHATE
IN RELATION TO HEALTH, SURVIVAL AND GENETIC FACTORS



natalia campos obando

BONE AND PHOSPHATE IN RELATION TO HEALTH, SURVIVAL AND GENETIC FACTORS

natalia campos obando

Cover: Yuliana Cruz Zuñiga
Lay-out: Marta Aguilar Díaz

ISBN: 978-9968-48-518-9

Bone and phosphate in relation to health, survival and genetic factors

Bot en fosfaat in relatie tot gezondheid, overleving en genetische factoren

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van der Rector Magnificus

Prof. Dr. R.C.M.E. Engels

en volgens het besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

donderdag 28 mei 2020 om 15:30 uur

door

natalia campos obando

geboren te **SAN JOSE, Costa Rica**

A DIOS,
Origen de todo bien y Fuente insondable de Misericordia

A la Inmaculada Virgen del Carmen y al Patriarca San José,
por su generoso Auxilio y Protección en todo momento

A mis padres,
por su amor y apoyo incondicional y por estar a mi lado siempre

To my Promotors,
Prof. Dr. MC Zillikens & Prof. Dr. AG Uitterlinden, for allowing me to become part of your wonderful group and for all your teachings

A mis queridos pacientes,
por sus muestras de apoyo y sus Oraciones.
Ustedes son la razón de ser de nosotros los médicos.

PROMOTIECOMISSIE

Promotoren:	Prof. Dr. M.C. Zillikens
	Prof. Dr. A.G. Uitterlinden
Overige leden:	Prof. Dr. Ewout Hoorn
	Prof. Dr. Paul Lips
	Prof. Dr. Bruno Stricker

CONTENTS

PROPOSITIONS OF THIS THESIS

Bone and phosphate in relation to health, survival and genetic factors

1. The relation between low bone mineral density and increased mortality in men is in part explained by higher mortality from chronic obstructive pulmonary disease.

[Looker, 2014 & this thesis]

2. Bone mineral density of the femoral neck, composed predominantly of cortical bone, is not causally related to arterial calcification.

[Schulz et al, 2004; Chow et al, 2008 & this thesis]

3. Increased serum phosphate levels within the normal range are causally related to coronary artery calcification in the general population, and, as such, must be considered a cardiovascular risk factor.

[Dhingra, 2007 & this thesis]

4. The associations we found between low bone mineral density and increased yet normal phosphate levels with decreased survival, lung emphysema, and coronary artery calcification (P), strongly resemble the premature ageing syndrome described in *Fgf23*$^{-/-}$ and *kl/kl* mouse phenocopies.

[Kuro-o et al, 1997; Shimada et al, 2004 & this thesis]

5. Regardless of kidney function, increased phosphate levels are related to fracture risk in men, especially at trabecular-enriched bones (such as vertebral bodies and wrist); this finding is partly attributable to a predominant synthesis of FGF23 at trabecular osteocytes.

[Pereira et al, 2009; Delgado-Calle et al, 2011 & this thesis]

6. In pregnancy and lactation associated osteoporosis, a persistent low bone mineral density should trigger investigation for secondary causes for osteoporosis, including potential underlying genetic factors.

[this thesis]

7. Bone metabolism plays an important role in the anti-ageing FGF23/Klotho axis, as indicated by osteocytes being the main source of FGF23, *Klotho* being expressed also in osteocytes, and our findings of low bone mineral density being related to decreased survival.

[Riminucci et al, 2003; Komaba et al, 2017; Kuro-o, 2018 & this thesis]

8. Additional evidence to support that bone metabolism plays an important role in ageing and survival is indicated by the findings of nitrogenated bisphosphonates being related to decreased mortality -mediated through a reduction in bone loss and independent of fracture prevention.

[Reid et al, 2020 & Bliuc et al, 2019]

9. The sex difference in the association of serum phosphate with several clinical cardiovascular outcomes is not explained by sex dimorphism in its genetic factors.

[Felsenfeld et al, 1999; Calvo et al, 1988; Kemi et al, 2006 & this thesis]

10. Entropy-based methods (ε) are required to disentangle the strong linkage disequilibrium of the Major Histocompatibility Complex (6p21.3) to identify causal variants in genetic association studies (such as in the GWAS of serum phosphate).

[Nothnagel et al, 2002; Hirata et al, 2019 & this thesis]

11. Very early-on global collaboration and sharing of clinical, epidemiological, and genetic data in local infectious disease outbreaks, can limit societal and economic consequences for all countries in potential pan-epidemic outbreaks.

PROPOSICIONES DE ESTA TESIS

Hueso y fosfato en relación con salud, supervivencia y factores genéticos

1. La relación entre baja densidad mineral ósea y aumento en la mortalidad en los hombres se explica parcialmente por una mayor mortalidad por enfermedad pulmonar obstructiva crónica.

[Looker, 2014 & esta tesis]

2. La densidad mineral ósea del cuello femoral, compuesta predominantemente de hueso cortical, no está causalmente relacionada con calcificación arterial.

[Schulz y otros, 2004; Chow et al, 2008 & esta tesis]

3. El nivel elevado de fosfato sérico - aún dentro del rango normal - está causalmente relacionado con calcificación de las arterias coronarias en la población general, y como tal, debe ser considerado un factor de riesgo cardiovascular.

[Dhingra, 2007 & esta tesis]

4. Las asociaciones que encontramos entre baja densidad mineral ósea y aumento en los niveles normales de fosfato - aún dentro del rango normal - con disminución de la supervivencia, enfisema pulmonar y calcificación de las arterias coronarias (P), se asemejan fuertemente al síndrome de envejecimiento prematuro descrito en fenocopias de ratones *Fgf23*$^{-/-}$ y *kl/kl*.

[Kuro-o et al, 1997; Shimada et al, 2004 & esta tesis]

5. Independientemente de la función renal, el aumento en los niveles de fosfato está asociado con riesgo de fractura en los hombres, especialmente en los huesos enriquecidos en componente trabecular (como los cuerpos vertebrales y el radio distal); este hallazgo es parcialmente atribuible a una síntesis predominante de FGF23 en los osteocitos trabeculares.

[Pereira et al, 2009; Delgado-Calle et al, 2011 & esta tesis]

6. En la osteoporosis asociada a embarazo y lactancia, la persistencia de una densidad mineral ósea disminuida debe desencadenar la investigación de factores secundarios de osteoporosis, incluyendo potenciales factores genéticos subyacentes.

[esta tesis]

7. El metabolismo óseo desempeña un papel importante en el eje anti-envejecimiento FGF23/Klotho, indicado por los siguientes hallazgos: los osteocitos son la fuente principal de FGF23, la expresión de *Klotho* también ocurre en osteocitos, y nuestros hallazgos de baja densidad mineral ósea asociados con disminución en la supervivencia.

[Riminucci et al, 2003; Komaba et al, 2017; Kuro-o, 2018 & esta tesis]

8. La evidencia adicional para apoyar que el metabolismo óseo tiene un papel importante en el envejecimiento y la supervivencia está basada en el hallazgo que los bifosfonatos nitrogenados están relacionados con disminución de la mortalidad - mediada por una reducción en la pérdida ósea e independientemente de la prevención de fracturas.

[Reid et al, 2020 y Bliuc et al, 2019]

9. La diferencia por sexo en la asociación de fosfato sérico con diversos resultados cardiovasculares clínicos no se explica por dimorfismo sexual en los factores genéticos del fosfato.

[Felsenfeld et al, 1999; Calvo et al, 1988; Kemi et al, 2006 & esta tesis]

10. Se requieren métodos basados en entropía (ε) para dilucidar el fuerte desequilibrio de ligamiento del Complejo Mayor de Histocompatibilidad (6p21.3) y así poder identificar las variantes causales en los estudios de asociación genética (como en el GWAS de fosfato sérico).

[Nothnagel y et al, 2002; Hirata et al, 2019 & esta tesis]

11. La implementación de colaboración temprana a nivel global y el intercambio de datos clínicos, epidemiológicos y genéticos durante los brotes locales de enfermedades infecciosas, pueden limitar las consecuencias sociales y económicas para todos los países en potenciales brotes de pandemias.

Some abbreviations implemented in this thesis:

BMD: bone mineral density
LS: lumbar spine
P: serum phosphate levels
CAC: coronary artery calcification
FN: femoral neck
COPD: chronic obstructive pulmonary disease
HR: hazard ratio
CVD: cardiovascular disease
RS: Rotterdam Study
CKD: chronic kidney disease
AC: arterial calcification
FGF23: fibroblast growth factor 23
MR: Mendelian Randomization

1 Introduction

Knowledge of bone disorders and bone-related traits have progressively evolved from a fracture perspective into a broader spectrum of effects and consequences beyond fracture incidence. This burst of knowledge has been enabled by the wide availability of tools that assess crucial aspects of bone, such as bone mineral density (1) and microarchitecture (2). Introduced in the 1970s, the quantification of bone mass through radiographic absorptiometry became possible and provided an estimate of cortical hand bone mass (3). However, the assessment of bone mineral density (BMD) through dual X-energy absorptiometry (DXA) (1988) has become the most widely applied tool for quantifying bone mass (4).

Although not exactly yielding a density – due to its two-dimensional perspective - (5) DXA assessments have led to a standardization of bone mass into categories of clinical importance, such as normal BMD, osteopenia and osteoporosis (6). The definition of osteoporosis by the World Health Organization (WHO) in 1991 (7, 8) already acknowledged not only the decrease in bone quantity, but also emphasized a more recent concept of bone quality as part of its strength and structure that when compromised, similar to bone mass, its ability to sustain trauma and avoid a fracture can decrease (9).

Parallel to this evolution in bone density-related concepts, several important discoveries have allowed the incorporation of bone tissue within physiologic axes of relevance for energy homeostasis, immunity and mineral balance, due to the discovery that bone cells can synthesize factors as osteocalcin (10), lipocalin 2 (11) and FGF23 (12-14). In particular, the description of the bone-derived hormone FGF23 has enabled the establishment of the “bone-kidney axis” (15-17), where bone tissue exerts a major regulatory role in physiology. This axis highlights the important role of bone tissue far beyond a mere structure that supports loading and allows locomotion.

The key role of FGF23 as a hormone exerting master control in phosphate homeostasis (12, 18, 19) - has probably responded to the imminent need of avoiding conditions of excess after the incorporation of phosphate into the bone

skeletons of vertebrate. Occurring in late Silurian or early Devonian Period, circa 540 Mya, the evolution from aragonite (CaCO3)-derived to hydroxyapatite-derived ($3Ca_3[PO_4]2Ca[OH]_2$; $Ca_{10}[PO_4]_6Ca[OH]_2$) skeletons took place (15). It has been postulated that this landmark transition was responsible for a decrease in bone solubility and therefore an increase in the stability required for the intense activity of vertebrates in comparison to their ancestors, as their high metabolic activity induces decreases in pH that would have dissolved bone were it totally calcitic instead of phosphatic (20).

Identified in 2000 in human families with severe hereditary hypophosphatemia (13), FGF23 became known as a potent phosphaturic hormone (13, 15) that depends on α-Klotho (21) as a cofactor to exert its classical effects in target tissues through its specific receptor, FGFR1c (19). It has been consistently shown that osteocytes, osteoblasts and bone lining cells (19, 22, 23) are the main source of FGF23 synthesis in health and also in disease - such as chronic kidney disease (CKD) across all its stages (24).

Therefore, bone is not merely a phosphate reservoir but importantly exerts a tight control on its homeostasis (**Figure 1**). This growing body of evidence has definitely placed phosphate as a bone trait –and not only phosphate: the statement is valid also for calcium, especially considering the recent description of FGF23 as a calciotropic hormone, exerting fine tuning of serum calcium levels (25). Therefore, the available evidence has also highlighted the key importance that bone tissue exerts in both health and disease.

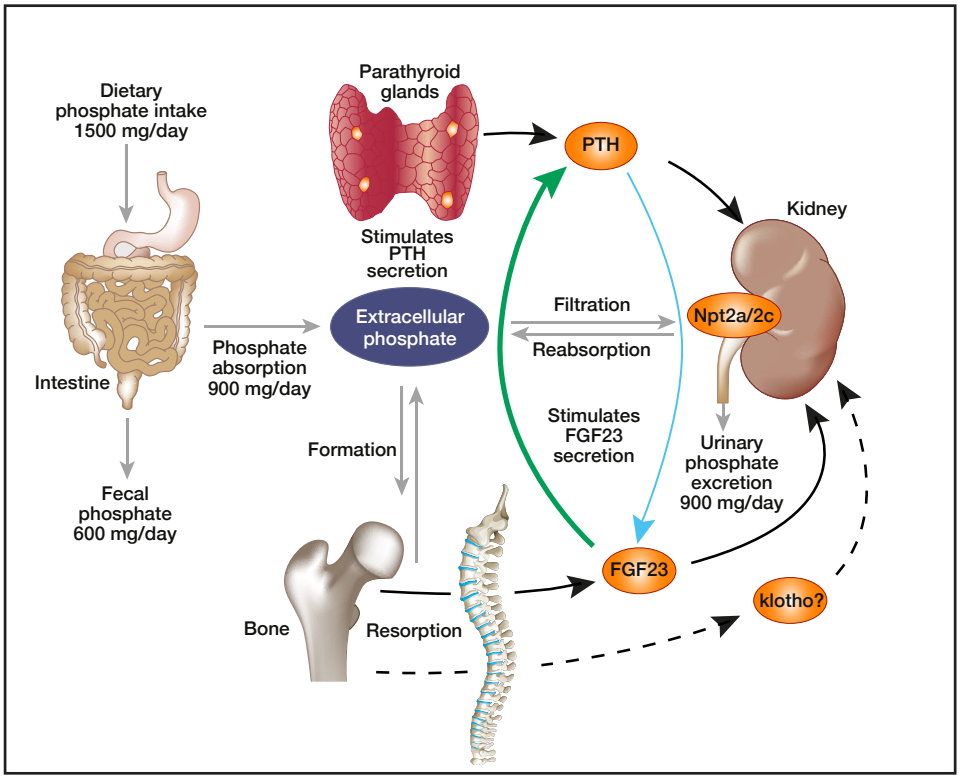


Figure 1. Serum phosphate levels homeostasis (modified from (26)). Continuous arrows show established axes; the intermittent arrows show a suggested axis; the red arrow shows negative homeostatic control. (Reproduced with permission from Copyright Clearance Center, Elsevier. Source (26))

Part I. Bone traits and phosphate in relation to health & survival

a. Previous studies on BMD beyond fracture incidence: impact on mortality

Aside from fracture incidence, one of the most important research questions studied in the context of low BMD conditions has been whether it has an impact on survival independently of shared risk factors for both mortality and BMD decrease. An initial perspective assigned to low bone density was that it only reflects the process of ageing and frailty (27-30), without an active role in it. This concept has been challenged since several studies carried out in different

populations have indeed described an independent association between low BMD and mortality (28, 31, 32). Nevertheless, previous research has not been able to identify a specific cause of mortality attributable to low BMD.

A putative relation of low BMD with cardiovascular disease (CVD) mortality has been based on the observation of a counterintuitive coexistence of vascular calcification and low BMD in several common clinical settings - condensed under the hypothesis of the "calcification paradox" (33) – where the presence of altered bone mineralization increases the pathological calcification in arterial walls (34). Due to the increase in CVD risk that vascular calcification entails (35), it has naturally been of great interest whether low BMD is related to CV events. Indeed, low BMD affecting CVD mortality was initially described in a large cohort of healthy-at-baseline women (29) but it has not been consistently reproduced throughout population-based cohorts (28, 31, 36). Though pooled evidence from the largest available meta-analysis provided evidence of a relation between low BMD and CV death (37), these findings were nullified due to publication bias (38). Similarly, an initial description of low BMD in association with stroke mortality (29) has not been reproduced in the analyses of pooled evidence (37, 39).

However, when a comprehensive classification of mortality causes has been applied, a systematic and strong relationship of low BMD with the residual non-CVD & non-cancer causes of mortality has been homogeneously reported across populations of different ethnic groups. It has therefore raised the question whether there is a specific (non-CVD & non-cancer) disease in which the condition of a low BMD impairs survival (28, 31, 40).

- ❖ BMD and mortality: are there sex differences?

In addition to the acknowledgment of a relationship between low BMD and mortality, evidence of a potential sex difference has progressively emerged. Data from NHANES (40), MrOS (31), SOF Research Group (29) and the Rotterdam Study (41) have clearly shown that survival in men is affected by a condition of low

BMD; but not so in women (40, 41). To date, no satisfactory explanation has been found to explain this difference. Nevertheless, despite these results from several cohorts, a consistent sex difference in the relationship between low BMD and mortality has not become manifest at the meta-analysis level (37).

❖ Low BMD and mortality in The Rotterdam Study: previous knowledge

In 2002, van der Klift and co-authors (41) published a prospective analysis on femoral neck BMD and all-cause mortality (in a follow-up period of 5.4 years) and demonstrated that men's survival was independently shortened by a condition of low BMD. Furthermore, the data provided evidence that the association was not linear, as high BMD values were also related to increased mortality in men; accordingly, the age-adjusted population average of BMD (Z=0) was found to be the safest value in terms of mortality, i.e., the BMD value not related to impaired survival. However, the authors did not find evidence that BMD influences survival in women.

These previous findings led us to formulate the following set of questions:

Q1a. *Is femoral neck BMD still related to all-cause mortality in a longer follow-up study?* ***Q1b****. Can we identify a specific disease whose survival is impaired by a condition of low BMD and explain this relationship, or is it all explained by fractures?*

BMD and the calcification paradox: rationale & how to analyze it in the Rotterdam Study

Independently of an association (or lack thereof) of BMD with mortality, the question of whether there is evidence of a joint occurrence of low BMD with vascular calcification (VC) beyond shared risk factors encloses importance for two main reasons: a) the prevalence of osteoporosis in the population is estimated for European countries to be 22% in women and 7% in men (42); and b) in the

general population, the presence of VC – more specifically, arterial calcification – is a major risk factor for cardiovascular events, such as myocardial infarction, stroke and heart failure (35, 43, 44). These two reasons highlight that the adverse effects of VC are definitely not confined to CKD (45).

In general, reports from the literature have not been consistent in the description of an association between BMD and arterial calcification (33). Nevertheless, longitudinal studies have found a relation between bone loss and arterial calcification progression in a more homogenous way (46, 47), especially in women. One of the most relevant circulatory beds for VC is at the coronary artery level, a process that mostly involves the intima (35, 48). Based on the available data on coronary calcification in the first cohort of the Rotterdam Study (**Figure 2**), we aimed to answer the following set of questions:

Q2a. *Is femoral neck BMD related to coronary artery calcification?* ***Q2b.*** *In addition, is femoral neck bone loss related to coronary artery calcification?* ***Q2c.*** *Is there evidence that the presence of coronary calcification increases fracture risk?*

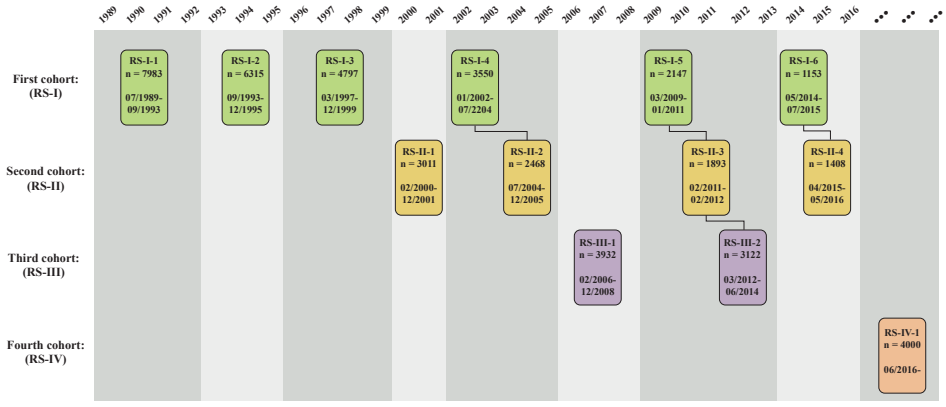


Figure 2. Schematic representation of the Rotterdam Study cohorts, including the baseline visit and the subsequent follow-up examination cycles.

❖ Serum phosphate levels (P) as a bone trait: rationale

Phosphorus is the most abundant anion in the human body and the second most common mineral (49). The majority of phosphorus is stored in bone and teeth (85%) and only 1% is present in extracellular fluids, either as organic or inorganic forms. Phosphate measured in serum (from now onwards P) corresponds to the amount of phosphorus in inorganic form (50).

Knowledge of an association of phosphorus with bone dates back more than 80 year ago, when Fuller Albright and his group described its crucial role in bone mineralization, as a condition of severely decreased phosphate levels – corresponding to the first description of X-linked hypophosphatemia - was found to induce a clinical picture of rickets with marked bone deformities, pain and multiple fractures in children. The status of defective mineralization was confirmed at the histologic level through a remarkable accumulation of wide osteoid seams without fibrosis (51). Not only is phosphorus an obligate substrate for hydroxyapatite formation: sustained hypophosphatemia has been shown to induce in mammals severe delays in the stages of osteocyte maturation and in the formation of secondary ossification centers (16, 17).

b. P and bone outcomes: BMD and fractures

Most reports and position statements focusing on the association between P and BMD and fractures have focused in the specific contexts where P levels lie in the extremes of its distribution: i.e., severe hyperphosphatemia and severe hypophosphatemia (52, 53). This probably reflects a tacit assumption of the lack of a relationship between normal P with bone outcomes.

The context of hypophosphatemia as risk factor for adverse skeletal outcomes is intuitive. As previously mentioned, P is an essential component of hydroxyapatite and exerts master control on osteocyte differentiation (16).

Though seemingly less intuitive, hyperphosphatemia has also been related to mineralization defects (14, 54), particularly in the setting of CKD (55), - where an altered bone mineral metabolism occurs partly in response to progressive P retention (15, 56). Indeed, hyperphosphatemia has been related to adverse bone consequences in both extremes of the spectrum of bone turnover:

a) Initially, a classic mechanism of severe secondary hyperparathyroidism (i.e., parathyroid hormone (PTH)-induced increased bone turnover) was described (57), characterized by increasing PTH in the course of CKD (58) and initially ascribed to decreased ionized calcium (57). Nevertheless, hyperphosphatemia is able per se to induce secondary hyperparathyroidism independently of serum levels of ionized calcium and 1,25-dihydroxyvitamin D (18, 59).

b) Currently, the opposite clinical setting of adynamic bone disease – a condition of extreme low bone turnover (60), - is also acknowledged to induce bursts in P and calcium as they cannot be normally incorporated into bone, leading to wide excursions in their serum levels (e.g., after food or medication intake) with subsequent adverse effects (61, 62).

Consistently, the impact of CKD-related hyperphosphatemia on bone metabolism has translated clinically into a substantially increased fracture risk (63).

In contrast, whether serum P levels are related to bone outcomes at the population level has been scarcely explored (64) and although some recent research has been performed between e.g., FGF23 levels and BMD (65), to the best of our knowledge a relation between BMD and P has not been studied. Motivated by the predominant role that bone-derived FGF23 has in P homeostasis - suggested by some studies as more important than PTH (58, 66) - and by the growing number of findings of normal P with adverse outcomes, we postulated the following set of questions:

***Q3a**. Is serum P related to BMD in the general population? Are there differences according to skeletal site? **Q3b.** Is P related to fracture risk –even within normal ranges? If so, can a particular pattern in fracture-site be identified? **Q3c.** Are there sex differences for any of these outcomes?*

P beyond bone: mortality as outcome

In (apparent) contrast to the impaired mineralization induced by hypophosphatemia, Block & Lowrie showed that marked hyperphosphatemia (>6.5 mg/dL) in patients with advanced CKD was associated with excess mortality (45, 67) due to a pathogenic mechanism of vascular calcification (VC). Recent research has revealed that VC is induced by calciprotein particles, in turn tightly related to P (68, 69). The risk of mortality in CKD patients without dialysis is also increased by P (70, 71).

More recently, moderate increases in P were found to be related to a higher mortality risk in patients with prevalent CVD (72). Finally, data from the Framingham Offspring Study revealed that an increasing yet normal P was associated with risk of CVD in both fatal and non-fatal events (73). The findings led the authors to raise an important question: Can a higher yet normal phosphate be actually considered a cardiovascular risk factor? (Dhingra et al, Framingham Offspring Study, 2007; (73)).

Subsequent studies have reproduced the Framingham Study findings (74, 75), and have found men as the only affected sex (75, 76). Furthermore, a report from NHANES III has replicated the finding that the impaired survival seems not to be driven by hyperphosphatemia, as the results have been found with predominantly normal P (76).

These cumulative findings have been considered to be important for public health by the European Food Safety Authority and by the European Commission (77, 78) as they concern P even within its normal range. In addition, the still unrestricted

phosphate intake in the population – especially from food additives – can not only increase time-average P levels but can also induce acute adverse effects, particularly in the cardiovascular and renal systems (68, 79).

Given these findings, we aimed to test for evidence of these associations within the Rotterdam Study, applying a few constraints directed towards improved inference, such as strictly normal P levels and exclusion of CKD patients. Our main set of questions are the following:

Q4a. *Is there evidence that higher - but within normal range - P increases all-cause mortality in data from Rotterdam? If so, what specific diseases are impaired by P, or is it driven by cardiovascular mortality? If not, is there evidence of P influencing other causes of mortality, as suggested from findings in animal studies?*
Q4b. *Are there sex differences in our results?*

❖ Is increasing but normal P a CV risk factor? Analysis to improve causal inference

Undoubtedly, from all possible questions concerning P & BMD in their association with health and disease, one of the most relevant was already raised by colleagues from Framingham more than 10 years ago (73). The (potential) establishment of increasing but normal P as a true CV risk factor would represent an important paradigm in the field of bone physiology, in epidemiology and, ideally, in public health policies. Therapeutically it would also mean a complex challenge to address.

We aimed therefore to answer this question and – eventually - provide a pathogenic mechanism underlying normal P levels and CV mortality. For such purposes, we selected coronary calcification as outcome due to its atherosclerotic nature, its ability to improve risk discrimination and the increased hazard for myocardial infarction, heart failure and death (35, 44) that it yields. Although severe hyperphosphatemia can induce arterial calcification of the media layer (80), whether this statement is also true for normal P in the intima layer has not

been explored from a causal perspective (81, 82).

To go further into causal inference, we aimed to apply Mendelian Randomization (MR), an Econometrics-derived technique based on the instrumentation of variables to avoid, or decrease, the chances of reverse causation and confounding (83, 84). P can be instrumented through its genetic variants (single nucleotide polymorphisms: SNPs) which genome-wide association studies (GWAS) have found to be associated with its levels (85, 86).

Using MR, we attempt to answer the following set of questions:

Q5a. *Are P serum levels causally related to coronary calcification? If so, are the results driven by hyperphosphatemia, CKD or prevalent CVD?* ***Q5b***. *Can we find evidence at the MR level of a sex-difference, strongly suggested by epidemiological papers on the association between P & coronary calcification, CVD morbidity and CVD mortality?*

Part II. Sex differences in calcium and phosphate levels

- ❖ P and adverse outcomes: sex differences

Further studies in the general population have not only widened the spectrum of outcomes associated with P beyond CVD risk, but have also systematically reported that men are, by far, more affected by (high / high-normal / normal) P-related consequences than women. The outcomes where such a sex dimorphism have been demonstrated include: all-cause mortality at the meta-analysis level (76, 87), subclinical atherosclerosis (75) and CKD progression (88) and possibly coronary artery disease (76).

Although from a statistical perspective the sex difference is evident, from a biological perspective it poses a significant challenge as women display higher P than men in the postmenopausal state (89) – a difference only partially mediated

through gonadal steroids and not through FGF23 (50, 90, 91). Higher P has been systematically reported in postmenopausal women than men of the same age, despite the findings of attenuated - or even absent - associations of P with adverse outcomes in women (75, 76, 92). Although there must be a complex homeostatic mechanism explaining this apparent paradox, to date none has been elucidated.

Based on data from the Rotterdam Study and from patients at Erasmus MC Hospital we attempt to answer the following set of questions:

Q6a. *Do the differences in calcium and P span several age categories? Are they explained by gonadal steroids?* ***Q6b****. Can we find evidence in our data of another potential mechanism underlying the sex differences in calcium and P levels?*

Part III. Genetic studies in relation to bone and phosphate

❖ Exploring unusual genetic causes for osteoporosis and fractures: a Mendelian approach

Advances in genotyping technology and in joint efforts from large Consortia have allowed a progressive identification of genetic variants – single nucleotide polymorphisms: SNPs - that determine the heritable fraction of BMD (93-95). Most of these variants are common in frequency and individually explain a tiny amount of the heritability, but jointly explain an increasing proportion of the variance due to ever larger genome-wide association studies. Currently, under an additive model the variance in BMD explained by the cumulative set of SNPs identified in well-powered studies reaches approximately 12% (95). Some heterogeneity has been described according to skeletal site and to sex; this is as expected due to physiological differences (94).

The current evidence supports BMD to be determined by a large number of variants with a small individual effect – i.e., the polygenic or infinitesimal model, originally proposed by Sir Ronald Fisher for quantitative traits such as height

(96). Nevertheless, knowledge from clinical settings has also shown the existence of Mendelian disorders (97) due to several genes harboring variants that when mutated induce large effects on BMD and fracture risk, highlighting that BMD genetic architecture is far from simple and fully defined.

We include in this thesis two reports of clinical cases of severe osteoporosis and fractures following Mendelian inheritance and whose clinical evolution has diverged from expected of a common low BMD context:

a) A cluster of five families with severe early-onset osteoporosis and fractures following an unusual X-linked inheritance pattern and in whom a genetic diagnosis of osteogenesis imperfecta could not be found.

b) A young woman with congenital unilateral blindness who suffered from severe back pain after first pregnancy and delivery. The clinical workup showed severe osteoporosis and multiple vertebral fractures. She had close relatives with a positive history of osteoporosis and fractures.

Through these clinical reports, we aim to answer the following set of questions:

Q7a*. What are the mutations underlying the phenotype of these patients? Are they lying in BMD annotated genes? If not, what additional evidence can be obtained to support causality?* ***Q7b****. What lessons useful for the common clinical practice can be learned from these cases?*

❖ Exploring the genetic determinants of human serum phosphate: a non-infinitesimal genome-wide approach through large-scale Biobanks

Knowledge of the genetic determinants of a trait is important for understanding underlying biology and it can help to identify therapeutic targets (98). In addition, this knowledge may help in diagnostics and patient stratification in the framework of personalized medicine. Specifically for P as a trait, the possibility of potential

new therapeutics should be emphasized not only because of the high prevalence of CKD as a growing public health problem - reaching a prevalence of 13% worldwide (99) - but also because of the increasing evidence of adverse effects described in a strictly normal P context. This is especially the case concerning CVD outcomes in men (75, 76) and potentially affecting a large, but as of yet, undefined fraction of the general population.

In contrast to approaches implemented for the identification of genetic determinants for Mendelian disorders, the genetic architecture of most complex traits is currently resolved by genome-wide, hypothesis-free approaches (100). This method is based on the interrogation between common SNPs – usually one at a time- and the phenotype, and is currently known as GWAS: genome-wide association study (101). Methods such as the assessment of populations stratification (102) and genomic control (103) have improved the replication of findings in comparison to the pre-GWAS era.

Currently, only two GWAS have been published on serum P levels in the general population: one on European ancestry (86), and one on Japanese ancestry (85). The former study included a meta-analysis involving ~16000 participants within the CHARGE Consortium and identified five loci influencing P levels (86). The latter involved a GWAS within BioBank Japan (104) and included ~42000 participants; it showed replication of previous findings on CHARGE Consortium and in addition, identified another seven loci (85).

These GWAS suggest a locus close to ALPL, which encodes for the enzyme alkaline phosphatase (AP), as the most strongly locus associated with P levels. ALP hydrolyzes inorganic pyrophosphate into P - raising intracellular P levels but the effect on serum P levels is not yet clear (86). Nevertheless, the fraction of P variance explained by the identified SNPs until now is still low (<5%) and the studies have not determined if there is a sex-specific architecture underlying P levels, as might be expected from biology and epidemiological data.

With the aim of identifying more genetic determinants of P levels, we have made use of the UK Biobank, a large genetic and phenotypic resource comprising half a million participants and available for research only in the last few years (105). The high degree of relatedness and stratification (106) of this cohort makes it an optimal candidate to apply mixed models rather than standard linear regression models, in order to avoid the exclusion of a large number of samples (~30%). Through conditioning on polygenic SNPs, mixed models can also increase the effective sample size (107).

Recent developments in mixed-models software (107) allow for the incorporation of the non-infinitesimal model, whose underlying and more realistic assumption is that traits are determined by a finite number (a few thousand) of causal loci (108). If indeed P genetic architecture follows this pattern, a substantial gain in power for loci discovery can be obtained.

Through a large-scale biobanks approach, we aim to answer the following set of questions:

***Q8a**. What are the common genetic determinants of P levels in humans?* ***Q8b.** Are we able to identify low frequency or rare genetic variants with large effects?* ***Q8c.** Is there evidence of a non-infinitesimal architecture for P?* ***Q8d.** Can we find evidence of a sex-dimorphism in the genetic structure for serum P levels?*

Outline of this thesis

This thesis aims to provide evidence to answer the postulated questions -and related queries- in as much detail as possible within the following structure:

In **Chapter 2**, a potential association between BMD measured at the femoral neck and all-cause and in detailed, cause-specific mortality is tested. In particular, we aim to carefully evaluate whether or not the burden attributable to fracture-related mortality underlies or not this relation.

In **Chapter 3**, we assess whether femoral neck BMD and femoral neck bone loss are related to coronary calcification scores. In addition, we test whether coronary calcification is prospectively related to fracture risk.

In **Chapter 4**, the tacit assumption of no association between P levels within the normal range and fracture risk is challenged. We proceed further to explore a potential association between P and BMD under normal conditions – with a possible meaning for P in physiologic regulation, site-specific fracture risk, and the effect that CKD has on the association of P with fracture risk.

In **Chapter 5**, we aim to replicate previous findings of an association of (normal) P with all-cause mortality but, in addition, we want to explore further whether all the relationship is driven by CVD mortality or if another disease (s) can be identified for which survival is also impaired by P, as suggested by animal studies.

In **Chapter 6**, we accomplish two main objectives: first, we assess whether there is a phenotypic relationship between serum P levels and coronary calcification scores. Secondly, we move deeper into causal inference by applying the Mendelian Randomization technique to the obtained results in order to conclude whether or not high-normal serum P levels are a CVD risk factor.

In **Chapter 7**, the influence of sex hormones and serum vitamin D levels in differences in calcium and phosphate levels between men and women is evaluated.

In **Chapter 8**, we assess whether the sex differences in calcium and phosphate levels are consistent across a wide range of ages.

In **Chapter 9**, a clinical and genetic study is presented, assessing five families with X-linked osteoporosis and early-onset fractures, in whom osteogenesis imperfecta has been excluded. This study is the result of the joint efforts of several research groups in the Netherlands and Germany.

In **Chapter 10**, the case of a young pregnant woman with severe osteoporosis and vertebral fracture risk is described to illustrate the clinical and genetic work-up performed in the context of a diagnosis of transient osteoporosis of pregnancy.

In **Chapter 11**, a large scale GWAS within the Biobanks is shown, to explore further the genetic variants that influence P levels in humans. We aim to test whether there is sex dimorphism in P genetic architecture; furthermore, we evaluate the role of the X-chromosome in serum P levels.

The general aim of these studies is that the outcome of this research will not only add to common knowledge concerning bone and P in relation to health, disease and genetic factors. It will also widen the clinical perspective of bone and bone diseases and provide a rational for more proactive pursuit of adequate bone health in our patients. We also aim to increase awareness of potential P-related adverse effects on human health. Finally, we hope to contribute to the important initiative from the European Food and Safety Agency to improve the health of the general population regarding the dietary intake of phosphate.

References

1. **Kanis JA, McCloskey EV, Johansson H, Oden A, Melton LJ, 3rd, Khaltaev N**. A reference standard for the description of osteoporosis. *Bone* 2008;42(3):467-75.

2. **Gonnelli S, Cepollaro C.** The use of ultrasound in the assessment of bone status. *J Endocrinol Invest* 2002;25(4):389-97.

3. **Versluis RG, Vismans FJ, van de Ven CM, Springer MP, Petri H.** Radiographic absorptiometry of the phalanges as a screening instrument to detect osteoporosis of the hip. *Acta Radiol* 1999;40(4):418-21.

4. **Lewiecki EM, Binkley N.** DXA: 30 years and counting: Introduction to the 30th anniversary issue. *Bone* 2017;104:1-3.

5. **Kroger H, Vainio P, Nieminen J, Kotaniemi A.** Comparison of different models for interpreting bone mineral density measurements using DXA and MRI technology. *Bone* 1995;17(2):157-9.

6. **Looker AC, Wahner HW, Dunn WL, Calvo MS, Harris TB, Heyse SP** et al. Updated data on proximal femur bone mineral levels of US adults. *Osteoporos Int* 1998;8(5):468-89.

7. **Kanis JA.** Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. *Osteoporos Int* 1994;4(6):368-81.

8. Consensus Development Conference: prophylaxis and treatment of osteoporosis. *Am J Med* 1991;90:914-5.

9. **Seeman E, Delmas PD.** Bone quality--the material and structural basis of bone strength and fragility. *N Engl J Med* 2006;354(21):2250-61.

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

11. **Villalvilla A, Garcia-Martin A, Largo R, Gualillo O, Herrero-Beaumont G, Gomez R.** The adipokine lipocalin-2 in the context of the osteoarthritic osteochondral junction. *Sci Rep* 2016;6:29243.

12. **Martin A, David V, Quarles LD.** Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* 2012;92(1):131-55.

13. **ADHR Consortium.** Autosomal dominant hypophosphatemic rickets is associated with mutations in FGF23. *Nat Genet* 2000;26:345-8.

14. **Shimada T, Kakitani M, Yamazaki Y, Hasegawa H, Takeuchi Y, Fujita T et al.** Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004;113(4):561-8.

15. **Hu MC, Shiizaki K, Kuro-o M, Moe OW.** Fibroblast growth factor 23 and Klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* 2013;75:503-33.

16. **Zhang R, Lu Y, Ye L, Yuan B, Yu S, Qin C et al.** Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J Bone Miner Res* 2011;26(5):1047-56.

17. **Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B et al.** Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* 2006;38(11):1310-5.

18. **Silver J, Naveh-Many T.** FGF-23 and secondary hyperparathyroidism in chronic kidney disease. *Nat Rev Nephrol* 2013;9(11):641-9.

19. **Vervloet M.** Renal and extrarenal effects of fibroblast growth factor 23. *Nat Rev Nephrol* 2019;15(2):109-20.

20. **Ruben JA, Bennett AA.** The Evolution of Bone. *Evolution* 1987;41(6):1187-97.

21. **Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K et al.** Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006;444(7120):770-4.

22. **Guo YC, Yuan Q.** Fibroblast growth factor 23 and bone mineralisation. *Int J Oral Sci* 2015;7(1):8-13.

23. **Clarke BL.** FGF23 regulation of phosphorus homeostasis is dependent on PTH. *Endocrinology* 2011;152(11):4016-8.

24. **Pereira RC, Juppner H, Azucena-Serrano CE, Yadin O, Salusky IB, Wesseling-Perry K.** Patterns of FGF-23, DMP1, and MEPE expression in patients with chronic kidney disease. *Bone* 2009;45(6):1161-8.

25. **Rodriguez-Ortiz ME, Rodriguez M.** FGF23 as a calciotropic hormone.

F1000Res 2015;4.

26. **Komaba H, Fukagawa M.** Phosphate-a poison for humans? *Kidney Int* 2016;90(4):753-63.

27. **Johansson C, Black D, Johnell O, Oden A, Mellstrom D.** Bone mineral density is a predictor of survival. *Calcif Tissue Int* 1998;63(3):190-6.

28. **Mussolino ME, Gillum RF.** Low bone mineral density and mortality in men and women: the Third National Health and Nutrition Examination Survey linked mortality file. *Ann Epidemiol* 2008;18(11):847-50.

29. **Browner WS, Seeley DG, Vogt TM, Cummings SR.** Non-trauma mortality in elderly women with low bone mineral density. Study of Osteoporotic Fractures Research Group. *Lancet* 1991;338(8763):355-8.

30. **Mussolino ME, Armenian HK.** Low bone mineral density, coronary heart disease, and stroke mortality in men and women: the Third National Health and Nutrition Examination Survey. *Ann Epidemiol* 2007;17(11):841-6.

31. **Johansson H, Oden A, Kanis J, McCloskey E, Lorentzon M, Ljunggren O et al.** Low bone mineral density is associated with increased mortality in elderly men: MrOS Sweden. *Osteoporos Int* 2011;22(5):1411-8.

32. **Suzuki T, Yoshida H.** Low bone mineral density at femoral neck is a predictor of increased mortality in elderly Japanese women. *Osteoporos Int* 2010;21(1):71-9.

33. **Persy V, D'Haese P.** Vascular calcification and bone disease: the calcification paradox. *Trends Mol Med* 2009;15(9):405-16.

34. **Neven E, Bashir-Dar R, Dams G, Behets GJ, Verhulst A, Elseviers M et al.** Disturbances in Bone Largely Predict Aortic Calcification in an Alternative Rat Model Developed to Study Both Vascular and Bone Pathology in Chronic Kidney Disease. *J Bone Miner Res* 2015;30(12):2313-24.

35. **Budoff MJ, Achenbach S, Blumenthal RS, Carr JJ, Goldin JG, Greenland P et al.** Assessment of coronary artery disease by cardiac computed tomography: a scientific statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology. *Circulation* 2006;114(16):1761-91.

36. **Trivedi DP, Khaw KT.** Bone mineral density at the hip predicts mortality in elderly men. *Osteoporos Int* 2001;12(4):259-65.

37. **Veronese N, Stubbs B, Crepaldi G, Solmi M, Cooper C, Harvey NC et al.** Relationship Between Low Bone Mineral Density and Fractures With Incident Cardiovascular Disease: A Systematic Review and Meta-Analysis. *J Bone Miner Res* 2017;32(5):1126-35.

38. **Duval S, Tweedie R.** Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 2000;56(2):455-63.

39. **Qu X, Huang X, Jin F, Wang H, Hao Y, Tang T et al.** Bone mineral density and all-cause, cardiovascular and stroke mortality: a meta-analysis of prospective cohort studies. *Int J Cardiol* 2013;166(2):385-93.

40. **Mussolino ME, Madans JH, Gillum RF.** Bone mineral density and mortality in women and men: the NHANES I epidemiologic follow-up study. *Ann Epidemiol* 2003;13(10):692-7.

41. **van der Klift M, Pols HA, Geleijnse JM, van der Kuip DA, Hofman A, De Laet CE.** Bone mineral density and mortality in elderly men and women: the Rotterdam Study. *Bone* 2002;30(4):643-8.

42. **International Osteoporosis Foundation.** Broken bones, broken lives: A roadmap to solve the fragility fracture crisis in Europe. 2018.

43. **Bos D, Portegies ML, van der Lugt A, Bos MJ, Koudstaal PJ, Hofman A et al.** Intracranial carotid artery atherosclerosis and the risk of stroke in whites: the Rotterdam Study. *JAMA Neurol* 2014;71(4):405-11.

44. **Leening MJ, Elias-Smale SE, Kavousi M, Felix JF, Deckers JW, Vliegenthart R et al.** Coronary calcification and the risk of heart failure in the elderly: the Rotterdam Study. *JACC Cardiovasc Imaging* 2012;5(9):874-80.

45. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK.** Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998;31(4):607-17.

46. **Kiel DP, Kauppila LI, Cupples LA, Hannan MT, O'Donnell CJ, Wilson PW.** Bone loss and the progression of abdominal aortic calcification over a 25 year period: the Framingham Heart Study. *Calcif Tissue Int* 2001;68(5):271-6.

47. **Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia JB.** Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. *Osteoporos Int* 2008;19(8):1161-6.

48. **Nakamura S, Ishibashi-Ueda H, Niizuma S, Yoshihara F, Horio T, Kawano Y.** Coronary calcification in patients with chronic kidney disease and coronary artery disease. *Clin J Am Soc Nephrol* 2009;4(12):1892-900.

49. **Goretti Penido M, Alon US.** Phosphate homeostasis and its role in bone health. *Pediatr Nephrol* 2012;27(11):2039-48.

50. **Koeppen BM, Stanton BA.** The Renal System. In Koeppen VM and Stanton BA. *Berne and Levy Physiology*. 2010;Sixth Edition:557-636.

51. **Albright F BA, Bloomberg E.** Rickets resistant to vitamin D therapy. *Am J Dis Child* 1937;54(3):529-47.

52. **KDIGO.** Clinical Practice Guideline Update for the diagnosis, evaluation, prevention and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl*. 2017;7(1):1-59.

53. **Haffner D, Emma F, Eastwood DM, Duplan MB, Bacchetta J, Schnabel D et al.** Clinical practice recommendations for the diagnosis and management of X-linked hypophosphataemia. *Nat Rev Nephrol* 2019;15(7):435-55.

54. **Ichikawa S, Imel EA, Kreiter ML, Yu X, Mackenzie DS, Sorenson AH et al**. A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Musculoskelet Neuronal Interact* 2007;7(4):318-9.

55. **Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K et al.** Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 2006;69(11):1945-53.

56. **Kuro OM, Moe OW.** FGF23-alphaKlotho as a paradigm for a kidney-bone network. Bone 2017;100:4-18.

57. **Silver J, Naveh-Many T.** Phosphate and the parathyroid. *Kidney Int* 2009;75(9):898-905.

58. **Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H et al.** Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011;79(12):1370-8.

59. **Cannata-Andia JB, Carrera F.** The Pathophysiology of Secondary Hyperparathyroidism and the Consequences of Uncontrolled Mineral Metabolism in Chronic Kidney Disease: The Role of COSMOS. *NDT Plus* 2008;1(Suppl 1):i2-i6.

60. **Brandenburg VM, Floege J.** Adynamic bone disease-bone and beyond. *NDT Plus* 2008;1(3):135-47.

61. **Cejka D, Kodras K, Bader T, Haas M.** Treatment of Hemodialysis-Associated Adynamic Bone Disease with Teriparatide (PTH1-34): A Pilot Study. *Kidney Blood Press Res* 2010;33(3):221-6.

62. **Coen G, Ballanti P, Mantella D, Manni M, Lippi B, Pierantozzi A et al.** Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. *Am J Nephrol* 2009;29(3):145-52.

63. **Pimentel A, Urena-Torres P, Zillikens MC, Bover J, Cohen-Solal M.** Fractures in patients with CKD-diagnosis, treatment, and prevention: a review by members of the European Calcified Tissue Society and the European Renal Association of Nephrology Dialysis and Transplantation. *Kidney Int* 2017;92(6):1343-55.

64. **Figueiredo CP, Rajamannan NM, Lopes JB, Caparbo VF, Takayama L, Kuroishi ME et al.** Serum phosphate and hip bone mineral density as additional factors for high vascular calcification scores in a community-dwelling: the Sao Paulo Ageing & Health Study (SPAH). *Bone* 2013;52(1):354-9.

65. **Jovanovich A, Buzkova P, Chonchol M, Robbins J, Fink HA, de Boer IH et al.** Fibroblast growth factor 23, bone mineral density, and risk of hip fracture among older adults: the cardiovascular health study. *J Clin Endocrinol Metab* 2013;98(8):3323-31.

66. **Ketteler M, Petermann AT.** Phosphate and FGF23 in early CKD: on how to tackle an invisible foe. *Nephrol Dial Transplant* 2011;26(8):2430-2.

67. **Lowrie EG, Lew NL.** Death risk in hemodialysis patients: the predictive value of commonly measured variables and an evaluation of death rate differences between facilities. *Am J Kidney Dis* 1990;15(5):458-82.

68. **Kuro-o M**. Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. *Nat Rev Nephrol* 2013;9(11):650-60.

69. **Miura Y, Iwazu Y, Shiizaki K, Akimoto T, Kotani K, Kurabayashi M et al.** Identification and quantification of plasma calciprotein particles with distinct physical properties in patients with chronic kidney disease. *Sci Rep* 2018;8(1):1256.

70. **Eddington H, Hoefield R, Sinha S, Chrysochou C, Lane B, Foley RN et al.** Serum phosphate and mortality in patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2010;5(12):2251-7.

71. **Kestenbaum B, Sampson JN, Rudser KD, Patterson DJ, Seliger SL, Young B et al.** Serum phosphate levels and mortality risk among people with chronic kidney disease. *J Am Soc Nephrol* 2005;16(2):520-8.

72. **Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G, Cholesterol and Recurrent Events Trial Inestigators.** Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005;112(17):2627-33.

73. **Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB, Sr., Gaziano JM et al.** Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007;167(9):879-85.

74. **Chang AR, Grams ME.** Serum phosphorus and mortality in the Third National Health and Nutrition Examination Survey (NHANES III): effect modification by fasting. *Am J Kidney Dis* 2014;64(4):567-73.

75. **Onufrak SJ, Bellasi A, Shaw LJ, Herzog CA, Cardarelli F, Wilson PW et al.** Phosphorus levels are associated with subclinical atherosclerosis in the general population. *Atherosclerosis* 2008;199(2):424-31.

76. **Onufrak SJ, Bellasi A, Cardarelli F, Vaccarino V, Muntner P, Shaw LJ et al.** Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009;169(1):67-77.

77. **European Food Safety Authority.** Assessment of one published review on health risks associated with phosphate additives in food. *EFSA Journal* 2013;11(11):3444, 27pp.doi:10.2903/j.efsa.2013.3444.

78. **Ritz E, Hahn K, Ketteler M, Kuhlmann MK, Mann J.** Phosphate ad-

ditives in food--a health risk. *Dtsch Arztebl Int* 2012;109(4):49-55.

79. **Chang AR, Anderson C.** Dietary Phosphorus Intake and the Kidney. *Annu Rev Nutr* 2017;37:321-46.

80. **Villa-Bellosta R, Millan A, Sorribas V.** Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am J Physiol Cell Physiol* 2011;300(1):C210-20.

81. **Shin S, Kim KJ, Chang HJ, Cho I, Kim YJ, Choi BW et al.** Impact of serum calcium and phosphate on coronary atherosclerosis detected by cardiac computed tomography. *Eur Heart J* 2012;33(22):2873-81.

82. **Gronhoj MH, Gerke O, Mickley H, Steffensen FH, Lambrechtsen J, Sand NPR et al.** Associations between calcium-phosphate metabolism and coronary artery calcification; a cross sectional study of a middle-aged general population. *Atherosclerosis* 2016;251:101-8.

83. **Burgess S, Thompson SG.** Use of allele scores as instrumental variables for Mendelian randomization. *Int J Epidemiol* 2013;42(4):1134-44.

84. **Imbens GW, Angrist JD.** Identification and estimation of local average effects. *Econometrica* 1994;62(2):467-76.

85. **Kanai M, Akiyama M, Takahashi A, Matoba N, Momozawa Y, Ikeda M et al.** Genetic analysis of quantitative traits in the Japanese population links cell types to complex human diseases. *Nat Genet* 2018;50(3):390-400.

86. **Kestenbaum B, Glazer NL, Kottgen A, Felix JF, Hwang SJ, Liu Y et al.** Common genetic variants associate with serum phosphorus concentration. *J Am Soc Nephrol* 2010;21(7):1223-32.

87. **Bai W, Li J, Liu J.** Serum phosphorus, cardiovascular and all-cause mortality in the general population: A meta-analysis. *Clin Chim Acta* 2016;461:76-82.

88. **Bellasi A, Mandreoli M, Baldrati L, Corradini M, Di Nicolo P, Malmusi G et al.** Chronic kidney disease progression and outcome according to serum phosphorus in mild-to-moderate kidney dysfunction. *Clin J Am Soc Nephrol* 2011;6(4):883-91.

89. **Keating FR, Jr., Jones JD, Elveback LR, Randall RV.** The relation of age and sex to distribution of values in healthy adults of serum calcium, inorganic phosphorus, magnesium, alkaline phosphatase, total proteins, albumin, and blood

urea. *J Lab Clin Med* 1969;73(5):825-34.

90. **Lederer E.** Regulation of serum phosphate. *J Physiol* 2014;592(18):3985-95.

91. **Meng J, Ohlsson C, Laughlin GA, Chonchol M, Wassel CL, Ljunggren O et al.** Associations of estradiol and testosterone with serum phosphorus in older men: the Osteoporotic Fractures in Men study. *Kidney Int* 2010;78(4):415-22.

92. **Yoo KD, Kang S, Choi Y, Yang SH, Heo NJ, Chin HJ et al.** Sex, Age, and the Association of Serum Phosphorus With All-Cause Mortality in Adults With Normal Kidney Function. *Am J Kidney Dis* 2016;67(1):79-88.

93. **Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB et al.** Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet* 2009;41(11):1199-206.

94. **Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, 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(5):491-501.

95. **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(10):1468-75.

96. **Fisher RA.** The Correlation between Relatives on the Supposition of Mendelian Inheritance. *Transactions of the Royal Society of Edinburgh* 1918;52:399-433.

97. **Mendel G.** Versuche uber Plflanzenhybriden. Verhandlungen der naturforschenden Vereines in Brunn, Bd. *IV fur das Jahr* 1865, Abhandlungen, 3-47.

98. **Plenge RM, Scolnick EM, Altshuler D.** Validating therapeutic targets through human genetics. *Nat Rev Drug Discov* 2013;12(8):581-94.

99. **Coresh J.** Update on the Burden of CKD. *J Am Soc Nephrol* 2017;28(4):1020-2.

100. **Risch N, Merikangas K.** The future of genetic studies of complex human

diseases. *Science* 1996;273(5281):1516-7.

101. **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* 2017;101(1):5-22.

102. **Patterson N, Price AL, Reich D.** Population structure and eigenanalysis. *PLoS Genet* 2006;2(12):e190.

103. **Devlin B, Roeder K.** Genomic control for association studies. *Biometrics* 1999;55(4):997-1004.

104. **Nagai A, Hirata M, Kamatani Y, Muto K, Matsuda K, Kiyohara Y et al.** Overview of the BioBank Japan Project: Study design and profile. *J Epidemiol* 2017;27(3S):S2-S8.

105. **Bycroft C, Freeman C, Petkova D, Band G, Elliott LT, Sharp K et al.** The UK Biobank resource with deep phenotyping and genomic data. *Nature* 2018;562(7726):203-9.

106. **Haworth S, Mitchell R, Corbin L, Wade KH, Dudding T, Budu-Aggrey A et al.** Apparent latent structure within the UK Biobank sample has implications for epidemiological analysis. *Nat Commun* 2019;10(1):333.

107. **Loh PR, Tucker G, Bulik-Sullivan BK, Vilhjalmsson BJ, Finucane HK, Salem RM et al.** Efficient Bayesian mixed-model analysis increases association power in large cohorts. *Nat Genet* 2015;47(3):284-90.

108. **Stahl EA, Wegmann D, Trynka G, Gutierrez-Achury J, Do R, Voight BF et al.** Bayesian inference analyses of the polygenic architecture of rheumatoid arthritis. *Nat Genet* 2012;44(5):483-9.

2

Bone Mineral Density and Chronic Lung Disease Mortality: The Rotterdam Study

Bone Mineral Density and Chronic Lung Disease Mortality: The Rotterdam Study

Authors:

Natalia Campos-Obando, Martha C. Castano-Betancourt, Ling Oei, Oscar H. Franco, Bruno H.Ch. Stricker, Guy G. Brusselle, Lies Lahousse, Albert Hofman, Henning Tiemeier, Fernando Rivadeneira, André G. Uitterlinden, M. Carola Zillikens

Status:

Published in *J Clin Endocrinol Metab* 2014; 99(5): 1834-42

Abstract

Context: Low bone mineral density (BMD) has been associated with increased all-cause mortality. Cause-specific mortality studies have been controversial.

Objective: The aim of the study was to investigate associations between BMD and all-cause mortality and in-depth cause-specific mortality.

Design and setting: We studied two cohorts from the prospective Rotterdam Study (RS), initiated in 1990 (RS-I) and 2000 (RS-II) with average follow-up of 17.1 (RS-I) and 10.2 (RS-II) years until January 2011. Baseline femoral neck BMD was analyzed in SD values. Deaths were classified according to International Classification of Diseases into seven groups: cardiovascular diseases, cancer, infections, external, dementia, chronic lung diseases, and other causes. Gender-stratified Cox and competing-risks models were adjusted for age, body mass index, and smoking.

Participants: The study included 5779 subjects from RS-I and 2055 from RS-II.

Main outcome measurements: We measured all-cause and cause-specific mortality.

Results: A significant inverse association between BMD and all-cause mortality was found in males [expressed as hazard ratio (95% confidence interval)]: RS-I, 1.07 (1.01-1.13), *P*=.020; RS-II, 1.31 (1.12-1.55), *P*=.001); but it was not found in females: RS-I, 1.05 (0.99-1.11), *P*=.098; RS-II, 0.91 (0.74-1.12), *P*=.362). An inverse association with chronic lung disease mortality was found in males [RS-I, 1.75 (1.34-2.29), *P* <.001; RS-II, 2.15 (1.05-4.42), *P*=.037) and in RS-I in females [1.72 (1.16-2.57), *P*=.008) persisting after multiple adjustments and excluding prevalent chronic obstructive pulmonary disease. A positive association between BMD and cancer mortality was detected in females in RS-I [0.89 (0.80-0.99); *P*=.043]. No association was found with cardiovascular mortality.

Conclusions: BMD is inversely associated with mortality. The strong association of BMD with chronic lung disease mortality is a novel finding that needs further analysis to clarify underlying mechanisms.

Osteoporosis is a condition characterized by low bone mineral density (BMD) and microarchitectural deterioration leading to decreased bone strength, which predisposes to fragility fractures and is associated with high morbidity and mortality (1). Low BMD has been linked to an increased risk of fracture-independent mortality and could be considered a predictor of survival (2,3). Regarding cause-specific mortality, studies on the association between BMD and cardiovascular disease (CVD) mortality have not been consistent, with some showing an inverse relation with BMD (2,4,5) whereas others found no association (6,7). Potential gender differences have been found both for both CVD mortality and cancer-related mortality, but results remain inconclusive (5,8,9). An inverse association between the group of remaining unspecified causes of mortality and BMD has been found in both genders (2,6).

Besides the association between low BMD and mortality, recent studies suggest that osteoporosis treatment reduces mortality, which could not be completely explained by a decrease in fracture-related mortality (10). Previously (3), we examined the relationship of femoral neck BMD with overall mortality in the first cohort of The Rotterdam Study (RS-I) and found an inverse association for all-cause mortality in men but not women after average follow-up of 5.4 years. The aim of this study was to analyze the relationship between BMD and all-cause and detailed cause-specific mortality in males and females from two cohorts of the Rotterdam Study with long-term follow-up.

Subjects and Methods

Study population

The Rotterdam Study is a prospective cohort study of males and females designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described previously (11). Inhabitants of the well-defined Ommoord district in the city of Rotterdam in The Netherlands were invited to participate; their names and addresses were drawn from the municipal

register. The RS-I, initiated in 1990, consisted of 7983 subjects; the second cohort (RS-II), initiated in 2000, included 3011 subjects. All participants were >55 years old at recruitment and reside in Ommoord, a district in Rotterdam. Analyses were performed in 5779 and 2055 subjects from RS-I and RS-II, respectively - all with available BMD measured at baseline and signed informed consent. The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus MC.

Dual-energy x-ray absorptiometry (DXA) scanning

BMD was assessed using DXA. Trained radiographic technicians performed BMD measurements for participants with a GE Lunar DPX-L densitometer (GE Lunar Corp), as previously described (12). Femoral neck BMD was chosen because it is not affected by degenerative changes seen with age as lumbar spine is, and it has been proposed for defining osteoporosis in epidemiologic studies (12,13).

Covariates

Several baseline covariates known to influence both BMD and mortality were included in the regression models, particularly age, gender, body mass index (BMI), and smoking status (14). BMI was calculated in kilograms per meter squared, from height and weight measured in a standing position without shoes. Smoking status was assessed by interview and coded as never, former, and current smokers. Other potential confounders were considered in additional analyses, such as physical activity, prevalent morbidity, medication, high-sensitivity C-reactive protein (hsCRP), and fracture incidence. For RS-I, a lower limb disability score was recorded using a modified version of the Stanford Health Assessment Questionnaire (15). For RS-II, a questionnaire on physical activity was collected. Baseline comorbidities were also assessed, such as prevalent myocardial infarction, dementia, type 2 diabetes mellitus, and chronic obstructive pulmonary disease (COPD); likewise, baseline medication use information was collected, such as bisphosphonate, hormone replacement therapy, and systemic corticosteroid use. Myocardial infarction prevalence was verified by a cardiologist,

a general practitioner (GP), or electrocardiogram. Prevalent COPD, diabetes mellitus, and dementia were defined as previously described (11,16). hsCRP was assessed as a marker of inflammation. Continuous covariates are described as mean and SD if there is normality of distribution; otherwise, median and interquartile range are shown. Fracture events were obtained from computerized records of GPs in the research area (covering 80% of the cohort); additionally, research physicians regularly followed participant information in the GPs' records outside the research area. All reported events were verified by two trained research physicians, who independently reviewed and coded the information. Finally, all coded events were reviewed by a medical expert for final classification according to the International Classification of Diseases, 10th revision (ICD-10) (17). Participants were followed from the baseline visit until January 1, 2007, or until a first fracture or death occurred. In addition, thoracolumbar spine radiographs were collected at both the baseline visit and the second follow-up visit (between 1997 and 1999). All thoracolumbar spine radiographs of the follow-up visit were scored morphometrically for the presence of vertebral fractures using the McCloskey/Kanis method (18). If a vertebral fracture was diagnosed, the baseline radiograph was also evaluated, and if present, it was considered a prevalent vertebral fracture. If it was not present at baseline, the fracture was considered as incident. Information on spinal radiographs was available for 3145 subjects with baseline DXA information.

Assessment of cause-specific mortality

Information on vital status is obtained continuously from the municipal authorities in Rotterdam. The cohorts are monitored for major disease outcomes and mortality through computerized linkage of the study database to GPs' medical files. For subjects who moved outside Ommoord, these data are obtained from GPs, who report all important events through a computerized system (3). Two research physicians independently coded the mortality events according to ICD-10 (19). Medical specialists in the respective field reviewed the coded events and confirmed the diagnosis. Information on cause-specific mortality was available

until January, 2011.

Different causes of mortality were recorded according to ICD-10 codes and first grouped into cardiovascular diseases (CVD), cancer, and "other causes" (5,6). To perform comprehensive analyses, the group of other causes was further categorized into external causes, dementia, infections, chronic lung disease, and other causes, following a slightly modified previous approach (8). This categorization yielded seven groups: CVDs (cardio- and cerebrovascular diseases; ICD10 codes I05-I30.0, I31, I32, I32.8, I34-I37, I42-I79, I81-I99, G95.1, F01), Cancer (codes C00-D09.9), chronic lung diseases (COPD comprised 80% of this group; codes J21.9, J39.9, J40, J42-J84.9, J90-J96), dementia (mostly Alzheimer's disease, codes F00, F02, F03, F05.1), external causes (mainly fatal fractures; mostly hip fractures, but also accidents and suicides; codes S00-T98 except T82.7), Infectious diseases (pneumonia and septic shock were the most common; codes A00-B99, G00-G09, I30.1, I32.0, I32.1, I33.0, J15-J18, J85-86, J41, K35-K38, K57.0, K75.0, I38-I41, I80, M00.9, M86, N71, T82.7) and other causes (heterogeneous group composed mainly of unspecified, unattended and sudden death and a minority of nontumoral gastrointestinal, renal, hematologic and cerebral diseases, senility and cachexia; codes D10-D48.9, E00-E90, D50-D89.9, G10-G26, K22-K34, K40-K52, K55.0, K56, K64, K73, K80, K83, K92, L12, L88, M05, M06, M31, M35, N03, N04, N10,N17, N18, N28, R54, R64, R96, R98, R99).

Statistical Analyses

For analysis of all-cause and cause-specific mortality, BMD was expressed in SD values, calculated as (patient BMD-cohort BMD mean)/cohort BMD SD, specific for gender. For RS-I, BMD was further categorized according to the number of SD (T-scores)- below the mean BMD for young adults (age, 20-29 y), leading to three strata: osteoporosis (T-score $\leq$ -2.5), osteopenia (-2.5 $<$ T-score $<$ -1.0), and normal BMD (T-score $\geq$ -1.0). Reference values for normal BMD were extracted from the Third National Health and Nutrition Examination Survey (NHANES III) for a non-Hispanic white population (20).

Table 1. Baseline Characteristics of the Participants of the Rotterdam Study with Femoral Neck BMD Measurement Available

	RS-I		RS-II	
	Males	**Females**	**Males**	**Females**
n	2438	3341	954	1101
Age, y	66.5 (61.3-72.7)[a]	67.5 (61.5-74.0)[a]	61.5 (8.9)[b]	61.4 (7.9)[b]
BMI, kg/m^2	25.6 (23.8-27.6)[a]	26.2 (23.9-29.1)[a]	26.7 (4.2)[b]	26.8 (5.8)[b]
FN BMD, g/cm^2	0.91 (0.82-1.00)[a]	0.82 (0.73-0.91)[a]	0.97 (0.13)[b]	0.89 (0.14)[b]
Never smokers, n (%)	197 (8)	1706 (51)	502 (53)	861 (78)
Former smokers, n (%)	1517 (62)	961 (29)	206 (22)	3(<0.1)
Current smokers, n (%)	711 (29)	646 (19)	243 (25)	234 (21)
Prevalent DM, n (%)	252 (10.3)	325 (9.7)	128(13.4)	112 (10.2)
Prevalent MI, n (%)	258 (10.6)	419 (12.5)	62 (6.5)	19 (1.7)

Abbreviations: FN, femoral neck; DM, diabetes mellitus; MI, myocardial infarction.
[a] Median (interquartile range).
[b] Mean (SD).

To assess the relation between BMD and mortality, Cox proportional hazard regressions were used, adjusting for age, BMI, and smoking. The proportional hazard assumption of the Cox models was assessed using the Schoenfeld residuals-based test - the standard diagnostic test. Both the P value for the BMD covariate itself and the P value for the global model were taken into account. For cause-specific mortality, additional analyses were performed running the competing-risk regressions based on the method of Fine and Gray (21) and taking into account informative censoring due to competing events. Proportionality was tested, evaluating interaction terms with time. All significant hazard ratios (HRs) reported here do not violate the proportionality assumption and thus are constant over follow-up time, unless stated otherwise. Analyses were sex-stratified because: 1) previous reports on gender differences have been described in the relation between BMD and mortality; and 2) Cox proportional hazards assumption violations were found due to gender. Because of fracture-related mortality (22), analyses were further adjusted for fracture incidence as a time-varying covariate. HRs with 95% confidence interval (CI) are expressed: 1) per decrease in SD of BMD; and 2) per category of osteopenia or osteoporosis, setting normal BMD as the reference. In a later step, analyses were repeated after exclusion of participants

with fatal events within the first 3 years, taking into account that low BMD might be a marker of underlying illness.

The association between BMD and cause-specific mortality was evaluated through cumulative incidence curves (CICs) instead of Kaplan Meier curves, which overestimate the risk probability when there are several types of possible events (21). CICs were calculated after running competing-risk regressions.

Results from individual cohorts were meta-analyzed. SPSS version 17 (SPSS Inc) and Stata version 12 (StataCorp) were used for the individual analyses. Comprehensive Meta-Analysis version 2.0 (Biostat) was used for the meta-analysis.

Results

All-cause mortality

In total, 5779 RS-I and 2055 RS-II subjects were followed for a median of 17.1 years and a mean of 10.2 years, respectively. During the follow-up, 3117 deaths occurred in RS-I and 295 in RS-II. Baseline characteristics for both cohorts are presented in Table 1. In both cohorts, a significant and inverse association between BMD and overall mortality was found in males, expressed as HR (95% confidence interval[CI]): RS-I, 1.07 (1.01-1.13), *P*=.020; RS-II, 1.31 (1.12-1.55), *P*= .001. In females, there was no association with overall mortality in either cohort. Adjustment for incident fractures yielded essentially the same results (data not shown).

Cause-specific mortality

A brief description of each cause and the frequencies are shown in Table 2. The HRs for BMD and cause-specific mortality adjusted for age, BMI, and smoking are shown in Table 3.

Table 2. Description and Frequencies of Cause-specific Mortality for Participants with BMD Available in RS-I and RS-II, up to 2011

Cause	Description	RS-I	RS-II
CVD	Cardio- and cerebrovascular pathology	1021 (32.8)	89 (30.1)
Cancer	All cancer-related deaths	829 (26.6)	110 (37.3)
Other causes	Non-cancerous gastrointestinal, hematological, cerebral and renal pathology; cachexia, senility, unattended, unspecified, and sudden death	640 (20.5)	45 (15.3)
Dementia	Dementia as final cause of death	241 (7.7)	18 (6.1)
Infectious diseases	All infectious-related deaths	162 (5.2)	12 (4.1)
Lung diseases	COPD, interstitial diseases, respiratory failure	121 (3.9)	13 (4.4)
External causes	Mainly hip fractures, accidents, suicides	91 (2.9)	7 (2.4)
Missing causes	Cases without ICD-10 codification	12 (0.4)	1 (0.3)
Total		3117 (100)	295 (100)

Data are expressed as number (percentage).

A relationship between lower BMD and higher chronic lung disease mortality was observed in RS-I and RS-II for males [RS-I, HR, 1.75 (95% CI, 1.34-2.29), $P <$ .001; RS-II, 2.15 (1.05-4.42), P=.037) and in RS-I for females [1.72 (1.16-2.57), P= .008]; whereas for females in RS-II, a similar but nonsignificant trend was observed [1.77 (0.28-11.2), P= .544) with only two deaths due to chronic lung disease. Most cases of chronic lung disease mortality were due to COPD (RS-I and RS-II combined, 104 of 134 cases, 77.6%), whereas the non-COPD cases were mainly due to interstitial pulmonary disease, pneumonitis, and unspecified respiratory failure. When we restricted the analyses to COPD mortality, the association between BMD and COPD mortality showed similar or even higher HRs than for chronic lung disease mortality (data not shown).

Table 3. All-Cause and Cause-Specific Mortality HRs for RS-I and RS-II per Decrease in SD of Femoral Neck BMD

	Males			Females		
	No. of Deaths	**HR (95%CI)**	***P***	**No of Deaths**	**HR (95%CI)**	***P***
All-cause						
RS-I	1488	**1.07** (1.01-1.13)	.020	1629	1.05 (0.99-1.11)	.098
RS-II	177	**1.31** (1.12-1.55)	.001	118	0.91 (0.74-1.12)	.362
Cardiovascular						
RS-I	507	0.97 (0.88-1.07)	.576	514	0.99 (0.90-1.09)	.865
RS-II	52	1.24 (0.91-1.67)	.168	37	0.91 (0.63-1.32)	.615
Cancer						
RS-I	438	1.04 (0.94-1.15)	.453	391	**0.89** (0.80-0.99)	.043
RS-II	66	1.29 (0.99-1.68)	.060	44	0.91 (0.65-1.27)	.598
Other causes						
RS-I	266	**1.18** (1.02-1.35)	.021	374	**1.21** (1.07-1.37)	.003
RS-II	27	1.25 (0.82-1.91)	.293	18	0.66 (0.41-1.07)	.092
Dementia						
RS-I	67	1.08 (0.82-1.42)	.594	174	1.20 (0.99-1.45)	.060
RS-II	09	1.83 (0.93-3.60)	.081	09	0.91 (0.40-2.08)	.822
Infections						
RS-I	84	1.14 (0.89-1.46)	.295	78	0.93 (0.72-1.20)	.572
RS-II	06	0.84 (0.41-1.74)	.650	06	2.51 (0.81-7.78)	.111
Chronic lung disease						
RS-I	81	**1.75** (1.34-2.29)	<.001	40	**1.72** (1.16-2.57)	.008
RS-II	11	**2.15** (1.05-4.42)	.037	02	1.77 (0.28-11.2)	.544
External causes						
RS-I	37	1.26 (0.86-1.82)	.231	54	**1.87** (1.30-2.68)	.001
RS-II	05	1.58 (0.59-4.23)	.361	02	0.96 (0.18-5.12)	.958

Adjustments were made for age, smoking, and BMI. Boldface data corresponds to hazard ratios with statistically significant P values.

Table 4. Chronic Lung Disease Mortality HRs per SD Decrease in Femoral Neck BMD in RS-I

	Males				**Females**			
	All Cases (n=81)		**Excluding Prevalent COPD (n=55)**		**All Cases (n=40)**		**Excluding Prevalent COPD (n=32)**	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Model I	**1.75** (1.33-2.29)	<.001	**1.55** (1.13-2.14)	.007	**1.72** (1.16-2.57)	.008	**1.79** (1.13-2.83)	.013
Model II	**1.73** (1.30-2.29)	< .001	**1.65** (1.16-2.33)	.005	**1.68** (1.12-2.54)	.012	**1.83** (1.14-2.93)	.013

Abbreviation: n, number of deaths. Model I was adjusted for age, BMI, and smoking. Model II was adjusted for age, BMI, smoking, lower limb disability, log CRP, baseline corticosteroid use, and prevalent and incident vertebral fractures. Boldface data corresponds to hazard ratios with statistically significant P values.

The HR magnitudes for chronic lung disease mortality were higher for males than females in both cohorts, and this difference remained after further adjustments for baseline medication use, such as bisphosphonates and hormone replacement therapy use (data not shown). Furthermore, adjustments for corticosteroid use, prevalent and incident radiographic and clinical vertebral fractures, log C-reactive protein (CRP) and lower limb disability score, and exclusion of COPD prevalent cases yielded similar results (Table 4). Figure 1A shows RS-I CICs (ie, the probability of dying of a chronic lung disease taking into account competing risks) for participants of median age, according to baseline BMD in SD values and adjusted for gender, BMI, lower limb disability, and smoking. Figure 1, B-D, shows the CICs stratified by smoking status in RS-I. Figure 1, E-H, shows correspondent CICs for RS-II.

There was a significant relationship between BMD and mortality due to other causes in RS-I [for males, HR, 1.18 (95% CI, 1.02-1.35), *P*=.021; for females 1.21 (1.07-1.37), *P*=.003]; whereas it was not significant in RS-II. Subanalysis of this group in RS-I attributed mortality mainly to unattended death, sudden death-cause unknown, and unspecified cause of mortality. For females in RS-I, there was a significant relationship between external causes of mortality and BMD [1.87 (1.30-2.68), *P*=.001], driven mainly by hip fracture mortality and a significant positive association between BMD and cancer mortality [0.89 (0.80-0.99), *P*= .043), whereas no associations were found in men.

There were no significant associations between BMD and mortality due to CVD, dementia, or infectious diseases.

Results from competing-risk regression models were similar to Cox models (data not shown).

The HRs from combined analysis of the two cohorts mostly reflected the results from the much larger RS-I cohort, both in all-cause and in cause-specific mortality analysis (data not shown).

Further adjustments for baseline comorbidity, bisphosphonate use, log CRP, and physical exercise did not substantially change the results in either cohort or gender (data not shown).

Risk of all-cause and cause-specific mortality in subjects with osteopenia and osteoporosis compared to those with normal BMD in RS-I

When analyzing the mortality in subjects with osteopenia and osteoporosis compared to those with normal BMD in RS-I, similar trends were found as with BMD in SD, except that females with osteoporosis also had increased risk of death. For all-cause mortality in RS-I, the HR for males with osteopenia was 1.26 (95% CI, 1.10-1.43; *P*< .001); and the HR for males with osteoporosis was 2.40 (1.39-4.16; *P*= .002), compared with those with normal BMD (Figure 2). Females with osteoporosis had 1.66 (1.17-2.35; P= .004) times increased risk of death. For chronic lung diseases mortality, males with osteopenia had 2.21 (1.34-3.62), *P*= .002) times increased risk, whereas for those with osteoporosis, the risk was increased 16.6 times (5.84-47.0), *P*< .001). Females with osteoporosis had 5.91 (1.20-29.0; *P*= .029) times increased risk for chronic lung diseases mortality. Figure 2, A and B, shows the HRs for all-cause mortality and mortality due to chronic lung disease in males and females with normal BMD, osteopenia, and osteoporosis. These analyses have been adjusted for age, BMI, and smoking. Further adjustments for incidental fractures yielded similar results (data not shown).

Exclusion of participants with early fatal events

Analyses excluding participants who died within the first 3 years of follow-up did not change results essentially (data not shown).

Discussion

In these analyses in two large prospective cohorts of elderly subjects from the Rotterdam Study, femoral neck BMD in SD was significantly inversely related

to overall mortality in males but not in females. This relationship was not driven by fracture-related mortality because adjustment for incident fractures yielded similar results. However, both males and females with osteoporosis had increased risk of death compared to those with normal BMD. When assessing cause-specific mortality in detail, we found a novel and inverse relation in both genders between BMD and mortality related to chronic lung diseases (mainly COPD).

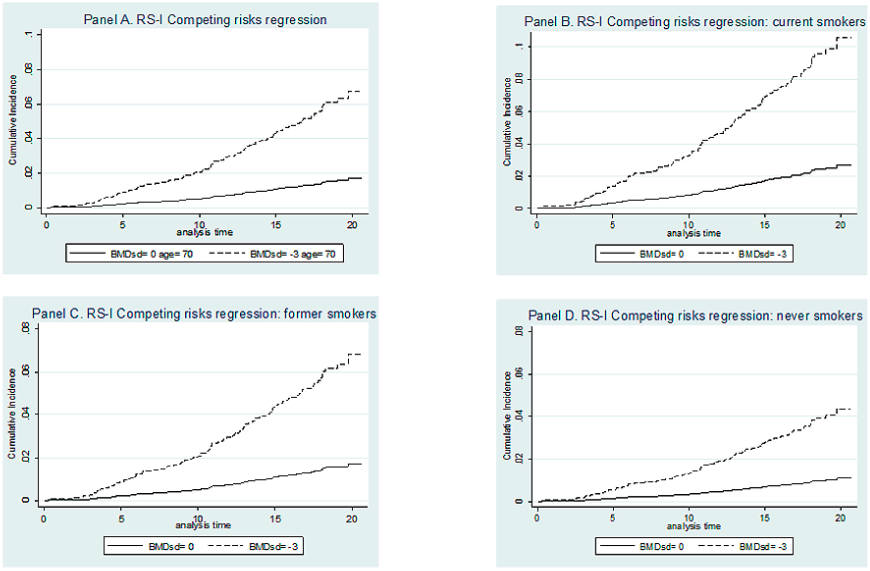


Figure 1. A, CIC for chronic lung disease mortality according to baseline BMD expressed in SD values (BMDsd), adjusted for gender, BMI, lower limb disability and smoking, for participants of median age of RS-I cohort. B-D, CIC according to baseline smoking status: current smokers (B), former smokers (C), and never smokers (D). The solid line represents participants with average BMD (BMDsd=0), and the dashed line represents participants with 3 SD below average BMD (BMDsd= -3). The analysis time is in years. E, CICs for chronic lung disease mortality according to baseline BMD expressed in BMDsd (BMDsd= -3). The analysis time is in years. E, CICs for chronic lung disease mortality according to baseline BMD expressed in BMDsd, adjusted for gender, BMI, physical activity, and smoking, for participants of median age of RS-II cohort. F-H, CIC according to baseline smoking status: current smokers (F), former smokers (G), and never smokers (H). The solid line represents participants with average BMD (BMDsd=0) and the dashed line represents participants with 3 SD below average BMD (BMDsd= -3). The analysis time is in years.

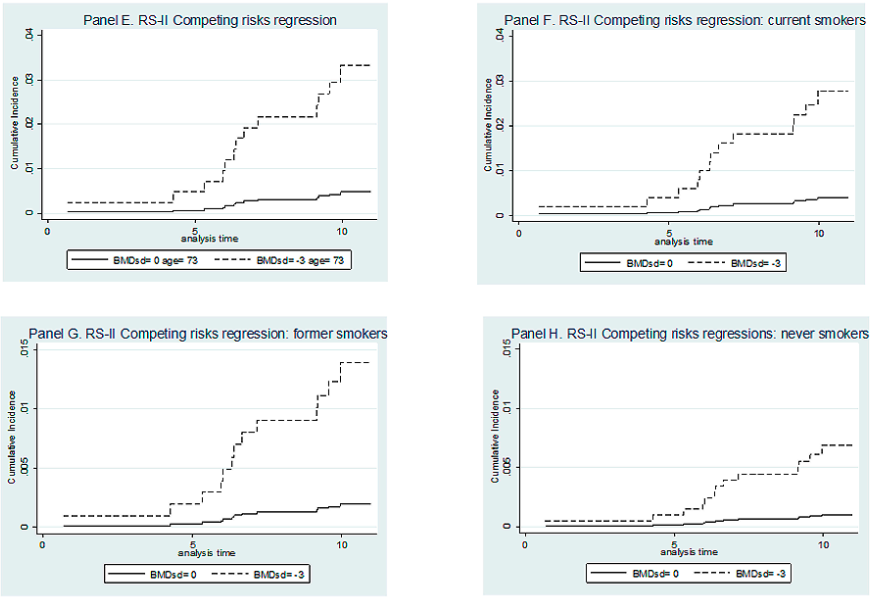


Figure 1. Continued.

In RS-I there was a significant inverse relationship between BMD and other causes of mortality in both genders, which was mainly observed in participants with unattended, unspecified, and sudden deaths. An inverse association of BMD with external causes of mortality (mainly due to hip fracture) was found in females in RS-I. No association was found between BMD and CVD mortality, dementia-related mortality, and death due to infectious diseases in either cohort. To the best of our knowledge, the relation we found between baseline BMD and chronic lung disease mortality has not been described before. The direction of association was consistent between genders and study cohorts and was not explained by prevalent COPD. Males and females with osteoporosis in RS-I had a 17 and 6 times increased risk of death due to chronic lung disease, respectively. It has been known that patients with COPD are at increased risk for low BMD and fractures (23,24), partly related to factors such as older age, smoking, physical inactivity, corticosteroid use, and low BMI (25), the latter being one of the more important determinants of low BMD according to a recent systematic review (26). All of these factors have

been taken into account in our analyses, and the HRs did not change, suggesting that other, yet unknown factors explain this relationship between BMD and COPD mortality. Chronic inflammation, associated to COPD, may induce low BMD, but additional adjustments for hsCRP did not modify the associations. Likewise, smoking, the most recognized environmental trigger for COPD (27), produces profound bone loss, mainly through increased osteoclast activity (28). Smoking induces IL-17 production, which is related to the development of emphysema (29) and is a potent inductor of inflammatory bone loss via stimulation of receptor activator of nuclear factor-κB ligand (30). Nevertheless, adjusting for smoking did not modify the association, and competing risk regression analyses showed similar associations in smokers as in nonsmokers.

Although vertebral fractures could compromise lung function and thus mortality (31,32), we did not find this to be an explanation for our findings because adjustment for prevalent and incident vertebral fractures did not modify results. Alternative explanations may be low vitamin D levels, which may lead to decreased BMD, as well as impaired lung function, or sarcopenia, and/or physical inactivity. COPD patients have a high prevalence of hypovitaminosis D and sarcopenia (33,34). Unfortunately, vitamin D levels were not available at baseline, but adjusting for disability index, which might be expected to associate with both low vitamin D and physical inactivity, did not change results. It was recently reported that the presence of anti-citrullinated protein antibodies is related to bone loss years before the occurrence of rheumatoid arthritis (35,36), while also being related to the development of airway abnormalities (37). This or other common factors might underlie our findings. Alternatively, a causal relationship between osteoporosis and (deaths from) other diseases like COPD cannot be ruled out since bone influences multiple other tissues and organs, eg, through its endocrine and immune-modulating properties as has been shown by multiple recent studies (38). Future studies should investigate a potential role of vitamin D, body composition, muscle strength, physical activity and potential common factors such as anti-citrullinated protein antibodies.

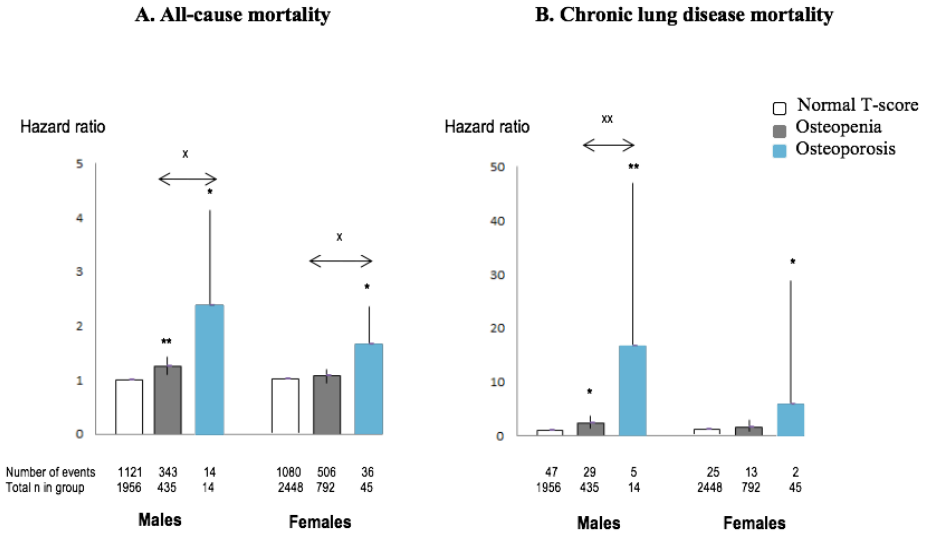


Figure 2. All-cause (A) and chronic lung disease (B) mortality HRs for participants from RS-I, according to T-score, adjusted for age, BMI, and smoking status. *, *P*< .05; **, *P*< .001 compared to normal T-score. x, *P*< .05; xx *P*< .001 osteoporosis compared to osteopenia.

Also, based on our findings it would be of public health importance to study further whether treatment of osteoporosis in patients with COPD would influence their mortality and potential pulmonary function.

In both studies, we found that femoral neck BMD was significantly inversely related to overall mortality in males but not in females. However, female subjects with osteoporosis also had increased risk of death. The relation between BMD and mortality in males was explained by an association with mortality from several causes, and similar findings were seen in females, except in the relation with cancer mortality. We found a decreased HR for cancer mortality with decreasing BMD in females, which may explain the absence of an association with overall mortality in females. A gender difference in cancer mortality was found in th NHANES I cohort (5), with a significantly increased HR for males but not females. Possible diverging associations between BMD and cancer mortality will have to be further investigated in well-powered studies.

We did not find a consistent association between BMD and CVD mortality, adding to the inconsistent literature findings of such a relationship. We found no association between BMD and dementia-related mortality. There is always the possibility that a low bone mass reflects a poor pre-existing health status, which may lead to increased mortality. We tried to account for this by excluding participants with mortality within the first 3 years of follow-up, but results were essentially the same.

This study has several limitations and strengths. A potential selection bias may have occurred since subjects who came to the research center at baseline for tests were healthier; however, if present, a dilution of the observed effects would be expected. Also, residual confounding cannot be excluded. Another weakness is that baseline COPD diagnosis was made based on clinical grounds and not in all cases on spirometry data, which is the "gold standard" method. Therefore, we cannot exclude the possibility of misclassification of COPD status at baseline, but it is not likely that this explains our finding. Also, we had no information on cumulative dose of corticosteroids in the past, only current use at baseline. Another potential limitation stems from the fact that the entire cohort is composed of European Caucasians, limiting the generalizability of our findings to other populations or ethnic groups.

One strength is the availability of two large prospective and similarly well-characterized, population-based prospective studies with accurate determination of causes of death. In general, data of The Netherlands registers is recognized as reliable and consistent (39), and more than 85% of the death registries in these cohorts were coded with high certainty level. As with any survival analyses, the completeness of follow-up is important, because when low it produces biased estimates (40). For both cohorts, the corresponding values for completeness of follow-up were higher than 90%, reassuring us that effect estimates obtained are valid.

In summary, we found an inverse relationship between BMD and all-cause mortality in males and an increased risk of death for male and female subjects with osteoporosis compared to those with normal BMD. This relationship was explained by several underlying causes, such as chronic lung disease mortality, trauma and other causes. A potential gender difference in the relation between BMD and cancer-related mortality with a positive association between BMD and cancer related mortality in females may explain the absence of an inverse relation between BMD and all-cause mortality in females. The consistent finding in both genders in both studies of an inverse and strong association between baseline BMD and chronic lung disease mortality has not been reported before and needs further study into the underlying pathophysiological mechanisms.

Acknowlegments- The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), The Netherlands Genomics Initiative (NGI), Netherlands Consortium of Healthy Ageing (NCHA), and the Municipality of Rotterdam. We thank the participants and staff of the research center of the Rotterdam Study.

Author contribution: Dr. Zillikens and N. Campos-Obando had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* N. Campos-Obando and dr. Zillikens. *Acquisition of data:* Prof. Hofman, Dr. Zillikens, dr. Rivadeneira, Prof. Uitterlinden, Prof. Stricker, Prof. Brusselle, L. Lahousse, Prof. Tiemeier. *Analysis and interpretation of data:* N. Campos-Obando and Dr. Zillikens. Drafting of the manuscript: N. Campos-Obando and Dr. Zillikens. *Critical revision of the manuscript for important intellectual content:* All authors. *Statistical analysis*: N. Campos-Obando. *Obtained funding:* Prof. Hofman, Prof. Uitterlinden. *Administrative, technical, and material support:* Dr. Zillikens, Prof Uitterlinden. *Study supervision:* Dr. Zillikens.

References

1. **Johnell O, Kanis JA.** An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int* 2006; 17(12):1726-1733.

2. **Johansson H, Odén A, Kanis JA, McCloskey E, Lorentzon M, Ljunggren Ö, Karlsson MK, Orwoll E, Tivesten Å, Ohlsson C, Mellström.** Low bone mineral density is associated with increase mortality in elderly men: MrOS Sweden. *Osteoporos Int* 2011; 22(5):1411-1418.

3. **van der Klift M, Pols HA, Geleijnse JM, van der Kuip DA, Hofman A, De Laet CE.** Bone mineral density and mortality in elderly men and women: the Rotterdam Study. *Bone* 2002 30(4):643-648.

4. **Qu X, Huang X, Jin F, Wang H, Hao Y, Tang T, Dai K.** Bone mineral density and all-cause, cardiovascular and stroke mortality: a meta-analysis of prospective cohort studies. *Int J Cardiol* 2013; 166(2): 385-393.

5. **Mussolino ME, Madans JH, Gillum RF.** Bone mineral density and mortality in women and men: the NHANES I epidemiologic follow-up study. *Ann Epidemiol* 2003; 13(10):692-697.

6. **Mussolino ME, Gillum RF.** Low bone mineral density and mortality in men and women: the Third National Health and Nutrition Examination Survey linked mortality file. *Ann Epidemiol* 2008; 18(11):847-850.

7. **Mussolino ME, Madans JH, Gillum RF.** Bone mineral density and stroke. *Stroke* 2003; 34(5):e20-22.

8. **Kado DM, Browner WS, Blackwell T, Gore R, Cummings SR.** Rate of bone loss is associated with mortality in older women: a prospective study. *J Bone Miner Res* 2000; 15(10):1974-1980.

9. **Ganry O, Baudoin C, Fardellone P, Peng J, Raverdy N.** Bone mass density and risk of breast cancer and survival in older women. *Eur J Epidemiol* 2004; 19(8):785-792.

10. **Bolland MJ, Grey AB, Gamble GD, Reid IR.** Effect of osteoporosis treatment on mortality: a meta-analysis. *J Clin Endocrinol Metab* 2010; 95(3):1174-1181.

11. **Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, Kuipers EJ, Nijsten TE, Stricker BH, Tiemeier H, Uitterlinden AG, Vernooij MW, Witteman JC.** The Rotterdam Study: 2012 objectives and design update. *Eur J Epidemiol* 2011; 26(8):657-686.

12. **Burger H, van Daele PL, Algra D, van den Ouweland FA, Grobbee DE, Hofman A, van Kuijk C, Schütte HE, Birkenhäger JC, Pols HA.** The association between age and bone mineral density in men and women aged 55 years and over: the Rotterdam Study. *Bone Miner* 1994; 25(1):1-13.

13. **Kanis JA, McCloskey EV, Johansson H, Oden A, Melton LJ 3rd, Khaltaev N.** A reference standard for the description of osteoporosis. *Bone* 2008; 42(3):467-475.

14. **Drake MT, Murad MH, Mauck KF, Lane MA, Undavalli C, Elraiyah T, Stuart LM, Prasad C, Shahrour A, Mullan RJ, Hazem A, Erwin PJ, Montori VM.** Clinical review. Risk factors for low bone mass-related fractures in men: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2002; 97(6):1861-1870.

15. **Pincus T, Summey JA, Soraci SA Jr., Wallston KA, Hummon NP.** Assessment of patient satisfaction in activities of daily living using a modified Stanford Health Assessment Questionnaire. *Arthritis Rheum* 1983; 26(11):1346-1353.

16. **van Durme YM, Verhamme KM, Stijnen T, van Rooij FJ, Van Pottelberge GR, Hofman A, Joos GF, Stricker BH, Brusselle GG.** Prevalence, incidence, and lifetime risk for the development of COPD in the elderly: the Rotterdam study. *Chest* 2009; 135(2):368-377.

17. International statistical classification of diseases and related problems, 10 th revision (ICD-10) Geneva: *WHO*, 1992.

18. **McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, Kanis JA.** The assessment of vertebral deformity: a method for use in population studies and clinical trials. *Osteoporos Int* 1993; 3: 138-147.

19. **Vliegenthart R, Oudkerk M, Hofman A, Oei HH, van Dijck W, van Rooij FJ, Witteman JC.** Coronary calcification improves cardiovascular risk prediction in the elderly. *Circulation* 2005; 112: 572-577.

20. **Looker AC, Wahner HW, Dunn WL, Calvo MS, Harris TB, Heyse SP , Johnston CC Jr, Lindsay R.** Updated data on proximal femur bone mineral levels of US adults. *Osteoporos Int* 1998; 8(5):468-489.

21. **Putter H, Fiocco M, Geskus RB.** Tutorial in biostatistics: competing risks and multi-state models. *Stat Med* 2007; 26(11):2389-2430.

22. **Haentjens P, Magaziner J, Colón-Emeric CS, Vanderschueren D, Milisen K, Velkeniers B, Boonen S.** Meta-analysis: excess mortality after hip fracture among older women and men. *Ann Intern Med* 2010; 152(6):380-390.

23. **Liang B, Feng Y.** The association of low bone mineral density with systemic inflammation in clinically stable COPD. *Endocrine* 2012; 42(1):190-195.

24. **Graat-Verboom L, van den Borne BE, Smeenk FW, Spruit MA, Wouters EF.** Osteoporosis in COPD outpatients based on bone mineral density and vertebral fractures. *J Bone Miner Res* 2011; 26(3):561-568.

25. **Biskobing DM.** COPD and osteoporosis. *Chest* 2002; 121(2):609-620.

26. **Graat-Verboom L, Wouters EF, Smeenk FW, van den Borne BE, Lunde R, Spruit MA.** Current status of research on osteoporosis in COPD: a systematic review. *Eur Respir J* 2009; 34(1):209-218.

27. **Balkissoon R, Lommatzsch S, Carolan B, Make B.** Chronic obstructive pulmonary disease: a concise review. *Med Clin North Am* 2011; 95(6):1125-1141.

28. **Yan C, Avadhani NG, Iqbal J.** The effects of smoke carcinogens on bone. *Curr Osteoporos Rep* 2011; 9(4):202-209.

29. **Chen K, Pociask DA, McAleer JP, Chan YR, Alcorn JF, Kreindler JL, Keyser MR, Shapiro SD, Houghton AM, Kolls JK, Zheng M.** IL-17RA is required for CCL2 expression, macrophage recruitment, and emphysema in response to cigarette smoke. *PLoS One* 2011; 6(5):e20333.

30. **Redlich K, Smolen JS.** Inflammatory bone loss: pathogenesis and therapeutic intervention. *Nat Rev Drug Discov* 2012; 11(3):234-250.

31. **Schlaich C, Minne HW, Bruckner T, Wagner G, Gebest HJ, Grunze M, Ziegler R, Leidig-Bruckner G.** Reduced pulmonary function in patients with spinal osteoporotic fractures. *Osteoporos Int* 1998; 8(3):261-267.

32. **Kado DM, Browner WS, Palermo L, Nevitt MC, Genant HK,**

Cummings SR. Vertebral fractures and mortality in older women: a prospective study. Study of Osteoporotic Fractures Research Group. *Arch Intern Med* 1999;159(11):1215-1220.

33. **Romme EA, Rutten EP, Smeenk FW, Spruit MA, Menheere PP, Wouters EF.** Vitamin D status is associated with bone mineral density and functional exercise capacity in patients with chronic obstructive pulmonary disease. *Ann Med* 2013; 45(1):91-96.

34. **Gelberg J, McIvor RA.** Overcoming gaps in the management of chronic obstructive pulmonary disease in older patients: new insights. *Drugs Aging* 2010; 27(5):367-375.

35. **Kleyer A, Finzel S, Rech J, Manger B, Krieter M, Faustini F, Araujo E, Hueber AJ, Harre U, Engelke K, Schett G.** Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. *Ann Rheum Dis Mar* 2013; 21.

36. **Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, Jakobsson PJ, Baum W, Nimmerjahn F, Szarka E, Sarmay G, Krumbholz G, Neumann E, Toes R, Scherer HU, Catrina AI, Klareskog L, Jurdic P, Schett G.** Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *J Clin Invest* 2012; 122(5):1791-1802.

37. **Demoruelle MK, Weisman MH, Simonian PL, Lynch DA, Sachs PB, Pedraza IF, Harrington AR, Kolfenbach JR, Striebich CC, Pham QN, Strickland CF, Petersen BD, Parish MC, Derber LA, Norris JM, Holers VM, Deane KD.** Brief report: airways abnormalities and rheumatoid arthritis-related autoantibodies in subjects without arthritis: early injury or initiating site of autoimmunity? *Arthritis Rheum* 2012; 64(6):1756-1761.

38. **Karsenty G, Ferron M.** The contribution of bone to whole-organism physiology. *Nature* 2012; 481(7381):314-320.

39. **Condran A, Himes C, SH P.** Old-age mortality patterns in low-mortality countries: an evaluation of population and death data at advanced ages, 1950 to the present. *Population Bulletin of the United Nations* 1991; 30:23-60.

40. **Clark TG, Altman DG, De Stavola BL.** Quantification of the completeness of follow-up. *Lancet* 2002; 359(9314):1309-1310.

3

Bone Health and Coronary Artery Calcification: The Rotterdam Study

Bone Health and Coronary Artery Calcification: The Rotterdam Study

Authors:

Natalia Campos-Obando*, Maryam Kavousi*,
Jeanine E. Roeters van Lennep, Fernando Rivadeneira,
Albert Hofman, André G. Uitterlinden,
Oscar H. Franco**, M. Carola Zillikens**

*These authors contribute equally to this work

** These authors jointly directed this work

Status:

Published in *Atherosclerosis* 2015; 241: 278-83

Abstract

Objectives*:* Vascular calcification has been associated inconsistently to low bone mineral density and fractures. The aims of the present study were to investigate the associations between coronary artery calcification (CAC) and BMD change, BMD and fracture risk in elderly subjects of the population-based Rotterdam Study.

Methods*:* BMD was assessed through dual-energy X-ray absorptiometry and CAC through Electron-Beam Computed Tomography in 582 men and 694 women. We investigated the associations between BMD change (6.4 years follow-up) and CAC at follow-up and between BMD and CAC (measured simultaneously). In sensitivity analyses we stratified analyses for estradiol levels in women. The association between CAC and fracture risk (9 years follow-up) was tested through competing-risks models. Models were sex-stratified and adjusted for age, body mass index, smoking, bisphosphonate use and age at menopause.

Results*:* There was no association between BMD change and CAC in men. In women, each 1% increase in annual BMD loss was significantly associated with higher follow-up CAC [β=0.22 (0.06 – 0.38), p=0.006; prevalence ratio: 4%]. Stratified analyses showed significant associations between BMD loss and follow-up CAC only in women with lower estradiol levels. We found no association between CAC and fracture risk and no association between BMD and CAC cross-sectionally.

Conclusions*:* BMD loss was associated with higher follow-up CAC in women, which might be related to low estrogen levels. No association between CAC and BMD or fracture risk was found. Further studies are required to elucidate the mechanisms that might underlie the association between BMD change and coronary calcification in women.

1. Introduction

Osteoporosis and cardiovascular disease (CVD) are common age-related diseases that have an increased co-existence independent of shared risk factors such as increased age, menopause, physical inactivity, alcohol intake and vitamin D deficiency [1]. Common pathophysiological mechanisms have been proposed such as inflammatory cytokines, oxidized lipids, increased homocysteine levels and decreased estrogen levels [1].

Vascular calcification is defined as the abnormal deposition of calcium in the vascular system [2]. Formerly considered a passive consequence of atherosclerosis, it is nowadays recognized as a highly active process associated with an increased risk of cardiovascular events independently of other traditional risk factors [3]. The resemblance that ectopic calcification shares with the normal calcification process of bone is remarkable and several studies [4,5] have verified the observation made by Virchow in 1863 that cardiovascular calcification is "an ossification, not a mere calcification". [6]

The increased co-existence of vascular calcification with osteoporosis [7] is called the calcification paradox. It has motivated several investigators to evaluate whether bone mineral density (BMD) and vascular calcification (VC) in several vascular beds are associated beyond the aging process and independent of potential confounders [8-14]. Among studies with a cross-sectional design, an inverse relation between aortic or coronary artery calcification (CAC) and BMD has been reported by some [8.9] but not others [10,11]. In contrast, longitudinal studies have consistently shown that increased BMD loss is associated with increased aortic vascular calcification assessed through different imaging modalities, such as X-rays and radiogrammetry [12,13] as well as through computed tomography [14], this relation has not been explained by aging and other shared risk factors and has been found mainly in women. Longitudinal studies evaluating the association between bone turnover and CAC have been performed mainly in subjects with chronic kidney disease, and results have been inconsistent; while some studies

have shown that low bone turnover is associated with increased risk of CAC [15] others have not replicated such findings [16].

Studies addressing the association between vascular calcification and fracture risk have focused mainly on aortic calcification, and the results have been conflicting. While some of them have reported an increased fracture risk with increased vascular calcification [14,17], other studies have not found such results [11,18].

Since previous studies found an association in women between BMD loss and aortic vascular calcification we aimed to investigate whether in the prospective population based Rotterdam study changes in BMD are associated with vascular calcification measured in the coronary arteries (CAC) in either sex and whether CAC is associated with incidental fractures and BMD. We also studied whether findings can be explained by hormonal status or bone turnover.

2. Materials and methods

2.1. Study population

The Rotterdam Study is a prospective cohort study of elderly men and women designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described elsewhere [19]. The Rotterdam Study I cohort (RS-I) was initiated in 1990 and consisted of 7983 participants. All subjects were >55 years at recruitment and reside in Ommoord, a district in Rotterdam and they have been assessed at baseline and through four follow-up visits. BMD was measured in all follow-up evaluations of the participants, and CAC scores were measured at RS-I-3 (third evaluation of the RS-I cohort). In total, 1276 subjects had available information on CAC levels, previous BMD measurements and incident fracture data (Fig. 1). The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus MC.

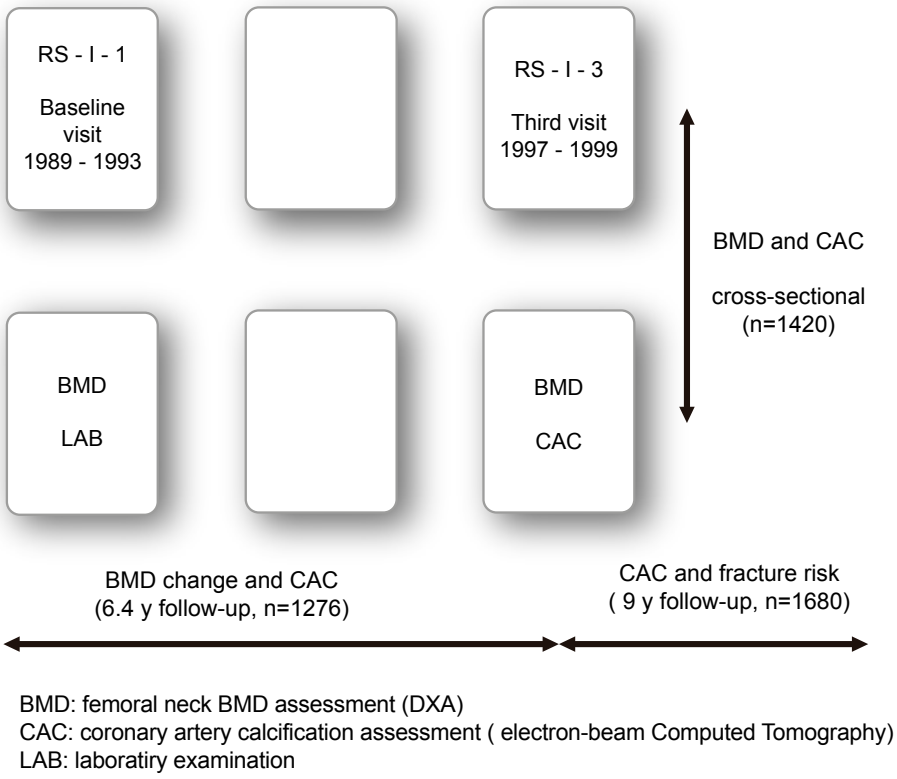


Figure 1. Flowchart for time line, design and sample size for the analyses

2.2. DXA scanning

BMD was assessed using dual-energy X-ray absorptiometry (DXA). Trained radiographic technicians performed BMD measurements for participants at the first visit (1990-1993) and the third visit (1997-1999) with a GE Lunar DPX-L densitometer. For the longitudinal analysis of BMD change and its association with follow-up CAC, absolute annual percent BMD change at the femoral neck was calculated with the formula [100 * (BMDRS-I-1 – BMDRS-I-3)/(BMDRS-I-1)*time length between measurements] [20], with a positive value reflecting BMD loss. Results are expressed per 1% increase in annual femoral neck BMD loss. Femoral neck BMD (from henceforth referred to simply as BMD) was chosen, as it is not affected by degenerative changes seen with age as lumbar spine BMD

and has been proposed for defining osteoporosis in epidemiologic studies [21]. For the cross-sectional analyses of BMD and CAC, BMD is expressed in sex-specific standard deviations (SD).

2.3. Coronary artery calcification assessment

At the third visit of the Rotterdam Study all participants who completed the third phase of the Rotterdam Study were invited to participate in the Rotterdam Coronary Calcification Study [22]. Epicardial coronary arteries calcification was detected by electron-beam Computed Tomography (EBT; C-150 Imatron Scanner, GE Healthcase, South San Francisco, CA). Before the subjects were scanned, they performed adequate breath-holding exercises. From the level of the root of the aorta through the heart, 38 images were obtained with a 100-ms scan time and a 3-mm slice thickness. During one breath hold, images were acquired at 80% of the cardiac cycle by using echocardiographic triggering. Quantification of coronary calcification was performed with AccuImage software (AccuImage Diagnostics Corporation, South San Francisco, CA) displaying all pixels with a density >130 Hounsfield Units (HU). The presence of calcification was defined as a minimum of 2 adjacent pixels (area=0.65 mm2) with a density > 130 HU. Calcium scores were calculated by multiplying the area in mm2 of individual calcified lesions with a factor based on the peak density of the lesion. The total calcification score for the entire epicardial coronary vascular system comprised the sum of the scores for all individual lesions.

2.4. Fracture assessment

Fracture events were obtained from computerized records of general practitioners (GPs) in the research area (covering 80% of the cohort); additionally research physicians regularly followed participant information in the GP's records outside the research area. All reported events were verified by two trained research physicians, who independently reviewed and coded the information. Finally, all coded events were reviewed by a medical expert for final classification according

to the International Classification of Diseases, tenth revision (ICD-10) [23]. Participants were followed from the date of the CAC scan until January 1, 2007, or until a first fracture or death occurred.

2.5. Covariates

Several covariates known to influence both BMD and coronary artery calcification scores (CACs) [4,24-26] were included in the regression models, particularly age, smoking, body mass index (BMI) and medication use (missingness <2%). BMI was calculated in kg/m2, from height and weight measured in standing position without shoes. BMI change was calculated as the absolute difference between measurements in the first and third visit of the Rotterdam Study. Smoking status was assessed by interview and coded as never-, former- and current smokers. Cigarette pack-years (for former and current smokers) were calculated as duration of smoking (in years) multiplied by the number of smoked cigarettes, divided by 20. Regarding medication use information, more than 99% of participants collected their drug prescriptions at seven regional pharmacies, which are fully computerized. Complete drug use information is available as of January 1st, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC) code from the World Health Organization (WHO) Collaboration Centre for Drug Statistics Methodology, the collection dates, total amount of drug units and product names of the drugs. Adjustments in our analyses were done for bisphosphonate [2] and hormone replacement therapy (HRT) use [27] due to the fact that both medication types have potential beneficial effects on vascular calcification. Bisphosphonate use was defined as exposure to the antiresorptive medication of at least 365 cumulative days before the date of the CAC scan. Further adjustments were done for serum lipid reducing therapy (mainly statins) and diuretic use, due to its effects on BMD and potential influence in coronary artery calcification [28].

Baseline comorbidity status was included in several models, namely prevalent diabetes mellitus, heart failure, peripheral artery disease and myocardial infarc-

tion; definition of such cases has been previously described elsewhere [29-32]. Laboratory covariates included in the analyses were 17β-estradiol (pmol/L) and alkaline phosphatase (missingness of 78% and 21% , respectively). For these measurements, non-fasting blood samples were drawn by venipuncture at the baseline visit between 0830 and 16 h. Platelets were removed by centrifugation and samples were stored at -80 C until measurements. 17β-estradiol (E2) was measured by direct immunoassay, and alkaline phosphatase (AP) was measured through an enzymatic colorimetric method. Other covariates included for further adjustments were total cholesterol, creatinine, 25-hydroxyvitamin D, serum calcium and phosphate levels, measured from blood samples obtained at baseline as previously described [19]. Intake of dietary calcium and Vitamin D was assessed by interview at baseline for food intake assessment using an extensive semi quantitative food frequency questionnaire (FFQ) at the study center by a trained dietician [19].

Additionally, analyses done for women were adjusted for age at menopause, collected by interview in the first visit.

2.6. Statistical analysis

Due to high skewness of the CAC measurements distribution that could not be completely corrected after log transformation, the association between BMD or BMD change and CAC scores was tested through generalized linear models, allowing Gaussian but also non-normal distributions for continuous variables. Log-transformed CAC scores (Ln(CAC+1)) were set as the dependent variable, with either BMD or BMD change as independent variables, adjusted for potential confounders. Fitness of different models was compared through the Akaike Information Criteria – AIC [33], models with lower values corresponding to a better fit. For assessment of the CAC score status in a binary fashion (yes/no), prevalence ratios were obtained with a *log* link instead of *logit* link, due to the fact that odds ratios overestimate the relative risks when the outcome is highly prevalent [34]. Assessments were made for BMD change and prevalent CAC at

the third visit of the Rotterdam Study, between CAC and subsequent fractures, and cross-sectionally for BMD and prevalent CAC both measured during the third visit (see Fig. 1).

As part of sensitivity analyses, we tested the significance of the interaction terms between BMD change with 17β-estradiol and alkaline phosphatase levels in those subsets with these measurements available (n=161 and n=556 with 17β-estradiol and alkaline phosphatase levels available, respectively) and performed stratified analysis according to 17β-estradiol (pmol/L) and alkaline phosphatase (U/L) levels, setting the cut-off point at the median value. Furthermore, analyses were performed after exclusion of participants with prevalent cardiovascular disease.

The association between CAC scores (at third visit) and incident fractures during follow-up was tested using competing-risks regression models which yield hazard ratio estimates and allow for informative censoring [35]. In this setting, the outcome of a fracture might not be seen because death occurs first, mainly because important risk factors for fracture incidence are shared for all-cause mortality [36]. For this analysis, the beginning of the follow-up period was set as the date of the CAC scan. The proportionality assumption was tested building interaction terms with time.

Analyses were performed with subjects with complete information on covariates, exposure and outcome.

SPSS (version 21.0, Armonk, NY: IBM Corp) and Stata (version 12, College Station TX: Stata Corp LP) were used for analyses. Statistical significance was defined as $p<0.05$.

3. Results

General characteristics of the population with information available on BMD change and CAC are displayed in Table 1. Age and BMI were similar between

men and women. Men had higher BMD, lower BMD loss rate, heavier smoking habits, and almost six-times higher CAC scores than women. CAC prevalence was high in both men and women (more than 85%).

The association between BMD change at the femoral neck (between baseline and third visit over an average of 6.4 y period) and follow-up CAC is depicted in Table 2. We found no significant associations in men [β= -0.02 (95%CI: -0.20 – 0.17), p=0.85); CAC prevalence ratio of 1%, p=0.16].

Table 1. General characteristics of the study population of 1276 men and women with information available on both BMD change and CAC

	Men (n=582)		**Women (n=694)**	
	Visit 1	**Visit 3**	**Visit 1**	**Visit 3**
Age (y)[a]	64.1 (59.9-68.2)	70.5 (66.4-74.9)	63.7 (59.8-68.2)	70.2 (66.3-74.7)
BMI (kg/m^2)[a]	25.9 (24.2-27.9)	26.2 (24.4-28.4)	25.9 (23.6-29.0)	26.8 (24.1-30.0)
BMD (g/cm^2)[b]	0.93 (0.13)	0.91 (0.13)	0.86 (0.13)	0.81 (0.13)
Annual FNBMD change (%)[a]	-	0.37 (-0.18-0.86)	-	0.78 (0.22-1.37)
Prevalent CAC (%)	n/a	569 (98%)	n/a	591 (85%)
CAC score[a]	n/a	271.5 (58.3-925.8)	n/a	48.7 (4.4-289.8)
Age at menopause[a] **(y)**	n/a	n/a	50.0 (46-52)	
Smoking (%)[c]	544 (93%)	535 (92%)	372 (54%)	369 (53%)
Prevalent CV disease[d] **(%)**	121 (21%)	-	94 (13%)	-

[a] Median and interquantile range

[b] Mean and standard deviation

[c] Current and former smokers

[d] Prevalent cardiovascular disease, defined as prevalent myocardial infarction, heart failure or peripheral artery disease

In women, we found that each 1% increase in annual BMD loss was significantly associated with higher CAC score on follow-up [β= 0.22 (0.06 – 0.38), p=0.006] and with higher CAC prevalence ratio of 4% (p=0.007). Adjustment for bisphosphonate use (n=48 users among a total of 1276 analysed subjects) did not

essentially change results. Additionally, adjustments for prevalent diabetes mellitus status, lipid lowering therapy (mainly statins) use, diuretic use, and levels of 25 hydroxyvitamin D, calcium, phosphate, creatinine and total cholesterol and dietary intake of calcium and vitamin D at baseline yielded similar results (data not shown). These "full-model" analyses were performed in a smaller subset of participants with available information in all mentioned covariates (n=235 men and n=290 women).

We investigated a potential relation between CAC scores and any type of fracture (total number of events=254; Table 3). We found no associations for any type of fracture incidence in either sex (Table 3).

We performed a cross-sectional analysis of BMD and CAC scores at the third visit (see Fig. 1), and found no association for either sex (men: β= -0.03 (-0.20 - 0.13), p=0.68; women: β= 0.01 (-0.16 - 0.19), p=0.89). Likewise, BMD was not associated with CAC prevalence in either sex in this cross-sectional analysis (Supplementary Table 1).

3.1. Sensitivity analysis

To further explore the association between BMD loss and follow-up CAC, we built interaction terms between BMD loss and two categories of respectively 17β-estradiol (E2) and alkaline phosphatase (AP) stratified by the median values (n=161 and n=556 women with E2 and AP measurements available, respectively). The *p* value results for both interaction terms were suggestive (p=0.13); therefore we proceeded to stratify the analysis of BMD loss and CAC by median level of E2 and AP. Table 4 shows that the associations between BMD loss and CAC seems to be confined to women with E2 levels below the median [β=0.55 (0.08-1.03), p=0.02] and to women with AP levels above the median [β=0.36 (0.12-0.60), p=0.003].

In addition, we investigated the influence of HRT use (n=119 HRT users) and prevalent CVD (n=96 women) on the relationship between BMD change and follow-up CAC in women, and the results remained robust after these additional analyses (data not shown).

4. Discussion

Overall we found that BMD loss (within an average period of 6.4 years follow-up) was significantly associated with higher follow-up CAC scores in women persisting after adjusting for multiple factors. This relationship was not observed for men, and we found no association of CAC scores with subsequent fractures in either sex.

Table 2. Annual percent BMD change at femoral neck and CAC scores in RS-I-3

	Model I			Model II		
CAC as continuous variable						
	n	***β* (95% CI)[a]**	***p***	**n**	***β* (95% CI)[a]**	***p***
Men	582	-0.02 (-0.20 - 0.17)	0.85	582	-0.02 (-0.21 – 0.17)	0.83
Women	694	**0.22** (0.06 – 0.38)	0.006	694	**0.23** (0.07 - 0.39)	0.005
CAC as binary variable[b]						
	n	**PR (95% CI)[c]**	***p***	**n**	**PR (95% CI)[c]**	***p***
Men	582	1.01 (0.99-1.02)	0.16	582	1.01 (0.99-1.02)	0.16
Women	694	**1.04** (1.01 – 1.07)	0.007	694	**1.04** (1.01-1.07)	0.007

Statistically significant results are highlighted in bold.

Model I: adjusted for age, BMI, delta BMI, smoking; in women also age at menopause. Model II: adjusted for covariates in Model I + bisphosphonate use before the date of the scan.

[a] β from linear regression for log CAC scores for 1% annual increase in BMD loss ($100^* [BMD_{RS\text{-}I\text{-}1} – BMD_{RS\text{-}I\text{-}3}]/[BMD_{RS\text{-}I\text{-}1}]$*time length between measurements)

[b] CAC binary refers to presence/absence of CAC. Present CAC is defined as a CAC score above 0.

[c] Prevalence ratio of CAC for 1% annual increase in BMD loss.

Table 3. Risk of incidence of all types of fracture as a function of CAC scores at RS-I-3 (third visit)

	Model I			Model II		
	no. of fxs	**HR (95% CI)[a]**	***p***	**n_o. of fxs**	**HR (95% CI)[a]**	***p***
All- fracture incidence						
Men	83/808	1.01 (0.90-1.14)	0.80	64/615	0.96 (0.86-1.08)	0.48
Women	171/872	1.02 (0.95-1.10)	0.48	124/655	0.99 (0.91-1.07)	0.75

Hazard ratios derived from competing-risks regression models.

Model I. Adjusted for age, BMI and smoking at RS-I-3.

Model II. Adjusted for covariates in Model I + BMD at RS-I-3.

[a] Hazard ratios expressed per increase in log CAC.

Our results are in line with three previous longitudinal studies that reported a significant association between BMD loss and vascular calcifications in the aorta in women [12-14] but associations of BMD change with CAC have not been reported in the general population to the best of our knowledge. We hereby describe for the first time an association with CAC in a general population setting of elderly (aged over 55 years). The association we found was not confounded by age, smoking, changes in BMI or bisphosphonate treatment.

The fact that BMD loss was associated with CAC among women only might suggest involvement of underlying hormonal factors as potential mechanisms. Exploratory stratified analyses showed that the association of BMD loss with CAC scores was observed in those women with lower baseline estradiol suggesting that low E2 levels could be involved in the development of both coronary calcification and BMD loss.

We found a significant association in the subgroup of women with higher AP levels, which may reflect higher bone turnover status induced by estradiol deficiency in the postmenopausal state [37]. AP induces the degradation of pyrophosphate (Pi), that plays a key role in ectopic calcification inhibition [38] that otherwise would occur in most tissues due to the fact that collagen, ubiquitously present, acts as a potent nucleating agent for the deposition of hydroxyapatite crystals [39]. The

increased AP levels in the postmenopausal state [40] may lead to lower Pi levels and therefore loss of inhibition of vascular calcification.

Vascular Smooth Muscle Cells (VSMC) can undergo differentiation towards an "osteoblast-like" phenotype, changing from a contractile to a synthetic state with subsequent secretion of extracellular matrix that eventually gets calcified [4,41]. There are several pathophysiological mechanisms that could explain the role that E2 plays in vascular calcification inhibition. In the first place, E2 prevents atherosclerotic plaque development [42], the only type of lesion that can get calcified in the coronary arteries as Mönckeberg's medial calcification does not occur in this vascular bed [43].

Table 4. Annual percent BMD change at femoral neck and CAC scores in RS-I-3 (third visit) in women stratified by baseline 17β-estradiol (E2) and alkaline phosphatase (AP) levels

	n	**β (95% CI)**[a]	***p***	**n**	**β (95% CI)**[a]	***p***
	E2 >16.4 pmol/L[b]			E2 <16.4 pmol/L[b]		
Women	81	-0.03 (-0.49 – 0.42)	0.88	80	0.55 (0.08 – 1.03)	0.02
	AP <76 U/L[c]			AP >76 U/L[c]		
Women	278	0.06 (-0.22 – 0.34)	0.68	278	0.36 (0.12 – 0.60)	0.003

Models adjusted for age, BMI, delta BMI, and smoking

[a] β from linear regression for log CAC scores for 1% annual increase in BMD loss (100* [$BMD_{RS\text{-}I\text{-}1}$ – $BMD_{RS\text{-}I\text{-}3}$]/[$BMD_{RS\text{-}I\text{-}1}$]*time length between measurements)

[b] E2 corresponds to baseline 17β-estradiol levels. Cut-off point was set at median value

[c] AP corresponds to baseline alkaline phosphatase levels. Cut-off point was set at median value

It has been previously shown that the administration of E2 decreases VSMC proliferation in animal and human models, through activation of nitric oxide synthase [44] and through decreased mitogen-induced VSMC proliferation. In second place, VSMC and endothelial cells express RANK, RANKL and OPG, and therefore can respond to RANKL stimulation. RANKL induces VC through an increase in bone morphogenetic protein 2 (BMP-2, the main stimuli of AP) and a decrease in matrix Gla protein (MGP), an inhibitor of VC. Importantly, E2 is able to attenuate RANKL-induced VC [42]. Therefore, through differential

actions in the expression of key proteins, E2 preserves the original contractile VSMC features, decreasing trans-differentiation towards a calcifying phenotype [44].

The beneficial effects of E2 on the coronary bed have been reported only in women [45]. This observation may explain the absence of a significant association between BMD loss and CAC in men in our study, despite the fact that BMD loss in the aging men is also associated with estradiol deficiency [46]. Consistent with our results, Kiel and colleagues previously described a lack of association between BMD loss and aortic calcification in men from the Framingham cohort [12].

We observed no different association between BMD loss and CAC regarding previous HRT use, suggesting that perhaps exogenous estradiol administration did not counterbalance the loss of atheroprotective effects associated with menopausal-related endogenous 17β-estradiol decrease. However, it should be emphasized that the age of HRT initiation or its duration in women from our cohort might not have been appropriate or long enough respectively to achieve a protective effect against coronary calcification, as the majority of HRT users reported a treatment length of less than 5 years and a previous RCT showed a beneficial effect of HRT after an average of 8.7 years of treatment in women aged 50-59 y at enrollment [27]. Nevertheless, it is important to mention that the effects of estrogen in the vascular system are complex and robust evidence has proven that in general HRT lacks sufficient beneficial effects on cardiovascular disease in both primary and secondary prevention settings in postmenopausal women [47].

Similar to other prospective studies performed in aortic calcification [11,18], we found no significant association between CAC and all-fracture risk in either sex during a mean follow-up of 9 years. This analysis takes risk of death into account. Of note, significant associations between aortic calcification and fractures have been previously [17] reported in studies with cross-sectional designs or with utilization of odds ratios as estimates of relative risks precluding determination

of causality for the calcification process on fracture risk. Furthermore, different devices in assessing bone mineral density, diversity in covariates adjusted for or different cohort characteristics might limit the comparability of results from multiple studies. We used electron-beam CT, which is a high sensible device to identify calcification and is superior to fluoroscopic measures; this is one of the strengths of our study.

Further strengths of our study include the prospective design in BMD change and fracture assessment with high completeness of follow-up [48] (more than 95%) that allows a better determination of how the natural history of disease might occur. The availability of several important confounders aid to decrease the bias introduced by risk factors that influence BMD loss and CAC. The assessment of longitudinal measurements of BMD using the same device avoided the need for calibration. The stratified analyses according to 17β-estradiol, AP levels and HRT use provide a deeper insight into the mechanisms and suggest that low estradiol levels may underlie both increased BMD loss and higher CAC but since these results derive from small subgroup analyses they require replication in larger (cohort) studies. There are other limitations. The analyses were performed in a subsample of the Rotterdam Study with available data on CAC measurement. However, despite some minor differences, characteristics of the responders to the Rotterdam Coronary Calcification Study were highly similar to those of the nonresponders [22]. Another limitation of the study is the lack of availability of PTH and FGF23 serum levels. Nevertheless, an association between long-term exposure of high PTH and vascular calcification has been demonstrated mainly in patients with renal dysfunction [49] and FGF23 does not seem to induce vascular calcification [50]. Despite multiple adjustments, residual confounding cannot be discarded. The fact that the entire cohort is composed of European Caucasians limits the generalizability of our findings to other populations or ethnic groups. Besides, the relatively short follow-up time available for the incidental fracture analysis might have limited our ability to detect an association between CAC and fracture risk. Furthermore, the stratified analysis according to E2 and AP levels were performed only in a small subset of women with such information available.

In conclusion, we found that BMD loss is significantly associated with higher CAC scores on follow-up in women only and we found no association between CAC levels and subsequent fractures. Our findings suggest that endogenous estradiol deficiency might underlie both pathological processes and thus be a shared risk factor for BMD loss and CAC but further studies are required to replicate these findings. Further research is warranted to explain the mechanisms that might underlie the association between BMD loss and CAC in women.

Role of the funding source

The funding sources had no role in the study design, collection, analysis or interpretation of data, in the writing of the report or in the decision to submit the article for publication.

Disclosures

Authors have no conflicts of interest.

Acknowlegments

The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw) (918.76.619), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), The Netherlands Genomics Initiative (NGI), Netherlands Consortium of Healthy Ageing (NCHA), and the Municipality of Rotterdam. Prof. Franco works in ErasmusAGE, funded by Nestlé Nutrition, Metagenics Inc. and AXA. Dr Kavousi is supported by AXA Research Fund.

References

1. **G.N. Farhat, J.A. Cauley**. The link between osteoporosis and cardiovascular disease. *Clin Cases Miner and Bone Metab* 2008 Jan;5(1):19-34.

2. **M. Wu, C. Rementer, C.M. Giachelli.** Vascular calcification: an update on mechanisms and challenges in treatment. *Calcif Tissue Int* 2013 Oct;93(4):365-73.

3. **M. Kavousi, S. Elias-Smale, J.H. Rutten et al**. Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study. *Ann Intern Med* 2012 Mar 20;156(6):438-44.

4. **N.X. Chen, S.M. Moe**. Vascular calcification: pathophysiology and risk factors. *Curr Hypertens Rep* 2012 Jun;14(3):228-37.

5. **L.L. Demer**. A skeleton in the atherosclerosis closet. *Circulation* 1995 Oct;92(8):2029-32.

6. **R. Virchow**. Cellular Pathology, as Based upon Physiological and Pathological Histology, Dover Publications, Inc, New York, NY, 1863, pp. 404-08.

7. **V. Persy, P. D'Haese**. Vascular calcification and bone disease: the calcification paradox. *Trends Mol Med* 2009 Sep;15(9):405-16.

8. **J.A. Hyder, M.A. Allison, N. Wong et al**. Association of coronary artery and aortic calcium with lumbar bone density: the MESA Abdominal Aortic Calcium Study. *Am J Epidemiol* 2009 Jan;169(2):186-

9. **S.H. Choi, J.H. An, S. Lim et al**. Lower bone mineral density is associated with higher coronary calcification and coronary plaque burdens by multidetector row coronary computed tomography in pre- and postmenopausal women. *Clin Endocrinol (Oxf)* 2009 Nov;71(5):644-51.

10. **B. Sinnott, I. Syed, A. Sevrukov, E. Barengolts**. Coronary calcification and osteoporosis in men and postmenopausal women are independent processes associated with aging. *Calcif Tissue Int* 2006 Apr; 78(4):195-202.

11. **E. Flipon, S. Liabeuf, P. Fardellone et al**. Is vascular calcification associated with bone mineral density and osteoporotic fractures in ambulatory, elderly women? *Osteoporos Int* 2012 May;23:1533-39.

12. **D.P. Kiel, L.I. Kauppila, L.A. Cupples, M.T. Hannan, C.J. O'Don-**

nell, P.W. Wilson. Bone loss and the progression of abdominal aortic calcification over a 25 year period: the Framingham Heart Study. *Calcif Tissue Int* 2001 May;68(5):271-76.

13. **A.E. Hak, H.A. Pols, A.M. van Hemert, A. Hofman, J.C. Witteman**. Progression of aortic calcification is associated with metacarpal bone loss during menopause: a population-based longitudinal study. *Arterioscler Thromb Vasc Biol* 2000 Aug;20(8):1926-31.

14. **E. Schulz, K. Arfai, X. Liu, J. Sayre, V. Gilsanz**. Aortic calcification and the risk of osteoporosis and fractures. *J Clin Endocrinol Metab* 2004 Sep;89(9):4246-53.

15. **D.V. Barreto, C. Barreto Fde, A.B. Carvalho et al**. Association of changes in bone remodeling and coronary calcification in hemodialysis patients: a prospective study. *Am J Kidney Dis* 2009; 52(6): 1139-50.

16. **G. Coen, P. Ballanti, D. Mantella et al**. Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. *Am J Nephrol* 2009; 29(3): 145-52.

17. **K.J. Kim, K.M. Kim, K.H. Park et al**. Aortic calcification and bone metabolism: the relationship between aortic calcification, BMD, vertebral fracture, 25-hydroxyvitamin D, and osteocalcin. *Calcif Tissue Int* 2012 Dec;91(6):370-78.

18. **E.J. Samelson, L.A. Cupples, K.E. Broe, M.T. Hannan, C.J. O'Donnell, D.P. Kiel**. Vascular calcification in middle age and long-term risk of hip fracture: the Framingham Study. *J Bone Miner Res* 2007 Sep;22(9):1449-54.

19. **A. Hofman, S. Darwish Murad, C.M. van Duijn et al**. The Rotterdam Study: 2014 objectives and design update. *Eur J Epidemiol* 2013 Nov;28(11):889-926.

20. **D.M. Kado, W.S. Browner, T. Blackwell, R. Gore, S.R. Cummings**. Rate of bone loss is associated with mortality in older women: a prospective study. *J Bone Miner Res* 2000 Oct;15(10):1974-80.

21. **J.A. Kanis, E.V. McCloskey, H. Johansson, A. Oden, L.J. Melton 3rd, N. Khaltaev**. A reference standard for the description of osteoporosis. *Bone* 2008 Mar;42(3):467-75.

22. **R. Vliegenthart, M. Oudkerk, A. Hofman et al**. Coronary calcification improves cardiovascular risk prediction in the elderly. *Circulation* 2005 Jul;112(4):572-77.

23. World Health Organization. International Statistical Classification of Diseases and Related Problems. 10th Revision (ICD-10). World Health Organization, Geneva, Switzerland, 1992.

24. **M.T. Drake, M.H. Murad, K.F. Mauck et al**. Clinical review. Risk factors for low bone mass-related fractures in men: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2012 Jun;97(6):1861-70.

25. **S. Elmariah, J.A. Delaney, K.D. O'Brien et al**. Bisphosphonate use and prevalence of valvular and vascular calcification in women MESA (The Muti-Ethnic Study of Atherosclerosis). *J Am Coll Cardiol* 2010;56(21):1725-29.

26. **J.C. Kovacic, P. Lee, U. Baber et al**. Inverse relationship between body mass index and coronary artery calcification in patients with clinically significant coronary lesions. *Atherosclerosis* 2012 Mar;221(1):176-82.

27. **J.E. Manson, M.A. Allison, J.E. Rossouw et al**. Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007 Jun;356(25):2591-2602.

28. **Q.O. Tang, G.T. Tran, Z. Gamie et al**. Statins: under investigation for increasing bone mineral density and augmenting fracture healing. *Expert Opin Invest Drugs* 2008 Oct;17(10):1435-63.

29. **A. Hofman, C.M. van Duijn, O.H. Franco et al**. The Rotterdam Study: 2012 objectives and design update. *Eur J Epidemiol* 2011 Aug;26(8):657-86.

30. **G.S. Bleumink, A.M. Knetsch, M.C. Sturkenboom et al**. Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure The Rotterdam Study. *Eur Heart J* 2004 Sep;25(18):1614-19.

31. **M. van der Klift, H.A. Pols, A.E. Hak, J.C. Witteman, A. Hofman, C.E. de Laet**. Bone mineral density and the risk of peripheral arterial disease: the Rotterdam Study. *Calcif Tissue Int* 2002 Jun;70(6):443-49.

32. **II de Liefde, M. van der Klift, C.E. de Laet CE, P.L. van Daele, A. Hofman, H.A. Pols**. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. *Osteoporos* Int 2005 Dec; 16(12): 1713-20.

33. **H. Akaike**. An information criterion (AIC). *Math Sci* 1976;14:5-9.

34. **S. Greenland**. Model-based estimation of relative risks and other epidemiologic measures in studies of common outcomes and in case-control studies. *Am J Epidemiol* 2004 Aug;160(4):301-5.

35. **H. Putter, M. Fiocco, R.B. Geskus**. Tutorial in biostatistics: competing risks and multi-state models. *Stat Med* 2007 May;26(11):2389-430.

36. **E. McCloskey, H. Johansson, A. Oden, J.A. Kanis**. Fracture risk assessment. *Clin Biochem* 2012 Aug;45(12):887-93.

37. **P.R. Ebeling, L.M. Atley, J.R. Guthrie et al**. Bone turnover markers and bone density across the menopausal transition. *J Clin Endocrinol Metab* 1996 Sep;81(9):3366-71.

38. **H. Fleisch, S. Bisaz**. Isolation fromo urine of pyrophosphate, a calcification inhibitor. *Am J Physiol* 1962 Oct;203(4):671-5.

39. **R.G. Russell**. Bisphosphonates: the first 40 years. *Bone* 2011 Jul;49(1):2-19.

40. **E. Romagnoli, G. Minisola, V. Carnevale et al**. Assessment of serum total and bone alkaline phosphatase measurement in clinical practice. *Clin Chem Lab Med* 1998 Mar; 36(3): 163-8.

41. **R.C. Johnson, J.A. Leopold, J. Loscalzo**. Vascular calcification: pathobiological mechanisms and clinical implications. *Circ Res* 2006 Nov;99(10):1044-59.

42. **M.K. Osako, H. Nakagami, N. Koibuchi et al**. Estrogen inhibits vascular calcification via vascular RANKL system: common mechanism of osteoporosis and vascular calcification. *Circ Res* 2010 Aug;107(4):466-475.

43. **M.J. Budoff, S. Achenbach, R.S. Blumenthal et al**. Assessment of coronary artery disease by cardiac computed tomography: a Scientific Statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology. *Circulation* 2006 Oct;114(16):1761-91.

44. **E. Rzewuska-Lech, M. Jayachandran, L.A. Fitzpatrick, V.M. Miller**. Differential effects of 17beta-estradiol and raloxifene on VSMC phenotype and expression of osteoblast-associated proteins. *Am J Physiol Endocrinol Metab*

2005 Jul;289(1):E105-12.

45. **P. Collins, G.M. Rosano, P.M. Sarrel et al**. 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. *Circulation* 1995 Jul 1;92(1): 24-30.

46. **M.T. Drake, S. Khosla**. Male osteoporosis. *Endocrinol Metab Clin North Am* 2012 Sep;41(3):629-41.

47. **J. Marjoribanks, C. Farquhar, H. Roberts, A. Lethaby**. Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2012 Jul;7:CD004143.

48. **T.G. Clark, D.G. Altman, B.L. De Stavola**. Quantification of the completeness of follow-up. *Lancet* 2002 Apr; 359(9314):1309-10.

49. **C. Goettsch, H. Iwata, E. Aikawa**. Parathyroid hormone: critical bridge between bone metabolism and cardiovascular disease. *Arterioscler Thromb Vasc Biol* 2014 Jul; 34(7): 1333-35.

50. **J.J. Scialla, W.L. Lau, M.P. Relly et al**. Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. *Kidney Int* 2013 Jun; 83(6): 1159-68.

Supplementary Material

Supplementary Table 1 Cross-sectional analysis of the association between femoral neck BMD and CAC in RS-I-3 (third visit)

	Model I			Model II		
CAC as continuous variable						
	n	β (95% CI)[a]	*P*	n	β (95% CI)[a]	*P*
Men	649	-0.03 (-0.20 – 0.13)	0.68	649	-0.04 (-0.20 – 0.13)	0.67
Women	771	0.01 (-0.16 – 0.19)	0.89	771	0.02 (-0.15 – 0.20)	0.79
CAC as binary variable[b]						
	n	PR (95% CI)[c]	*P*	n	PR (95% CI)c	*P*
Men	649	1.00 (0.99 – 1.01)	0.83	649	1.00 (0.99 – 1.01)	0.83
Women	771	1.00 (0.97 – 1.03)	0.95	771	1.00 (0.97 – 1.03)	0.99

[a] β from linear regression for log CAC scores for each increase in standard deviation of FN-BMD

[b] CAC binary refers to presence/absence of CAC. Present CAC is defined as a CAC score above 0

[c] Prevalence ratio of CAC for each increase in standard deviation of FN-BMD

Model I: adjusted for age, BMI, smoking; in women also age at menopause

Model II: adjusted for covariates in Model I + bisphosphonate use before the date of the CAC scan

4

Serum Phosphate Is Associated With Fracture Risk: The Rotterdam Study and MrOS

Serum Phosphate Is Associated With Fracture Risk: The Rotterdam Study and MrOS

Authors:

Natalia Campos-Obando*, W Nadia H Koek*, Elizabeth R Hooker,
Bram CJ van der Eerden, Huibert A Pols,
Albert Hofman, Johannes PTM van Leeuwen,
Andre G Uitterlinden, Carrie M Nielson,
and M. Carola Zillikens

*These authors contribute equally to this work

Status:

Published in *J Bone Miner Res* 2017; 32(6): 1182-93

Abstract

Extreme phosphate levels (P) have been associated with mineralization defects and increased fracture risk. Whether P within normal range is related to bone health in the general population is not well understood. To investigate the association of P with bone mineral density (BMD) and fracture risk, we assessed two population-based cohorts: the Dutch Rotterdam Study (RS-I, RS-II, RS-III; $n = 6791$) and the US Osteoporotic Fractures in Men (MrOS; $n = 5425$) study. The relationship of P with lumbar spine (LS) and femoral neck (FN) BMD was tested in all cohorts via linear models; fracture risk was tested in RS-I, RS-II, and MrOS through Cox models, after follow-up of 8.6, 6.6, and 10.9 years, respectively. Adjustments were made for age, body mass index, smoking, serum levels of calcium, potassium, 25-hydroxyvitamin D, estimated glomerular filtration rate (eGFR), FN-BMD, prevalent diabetes, and cardiovascular disease. Additional adjustments were made for phosphate intake, parathyroid hormone, and fibroblast growth factor 23 levels in MrOS. We further stratified by eGFR. Results were pooled through study-level metaanalyses. Hazard ratios (HR) and betas (β) (from meta-analyses) are expressed per 1 mg/dL P increase. P was positively associated with fracture risk in men and women from RS, and findings were replicated in MrOS (pooled HR all [95% CI]: 1.47 [1.31–1.65]). P was associated with fracture risk in subjects without chronic kidney disease (CKD): all (1.44 [1.26–1.63]) and in men with CKD (1.93 [1.42–2.62]). P was inversely related to LS-BMD in men (β: –0.06 [–0.11 to –0.02]) and not to FN-BMD in either sex. In summary, serum P was positively related to fracture risk independently from BMD and phosphate intake after adjustments for potential confounders. P and LS-BMD were negatively related in men. Our findings suggest that increased P levels even within normal range might be deleterious for bone health in the normal population.

Introduction

Phosphorus is the main mineral in the bone, where it is deposited together with calcium.[1] The intracellular compartment contains approximately 14% of phosphorus, and only 1% circulates freely in plasma as phosphate (P).[2] Within bone, phosphorus accumulates in the form of hydroxyapatite.[3] Phosphorus bioavailability is crucial for appropriate mineralization; [4] conditions of low phosphate are characterized by defective mineralization and excessive amount of unmineralized bone, or osteoid, typical of rickets/osteomalacia.[5,6] On the other hand, extreme hyperphosphatemia induces tumoral calcinosis, characterized by ectopic calcifications but also by mineralization defects.[7–9]

The recent finding that P regulation is exerted also by the phosphatonins a-Klotho and the osteocyte-derived fibroblast growth factor 23 (FGF23)[7,10] has established the concept that bone is not only a P reservoir but also acts as an endocrine organ,[3] regulating P levels and mineralization. Therefore, a potential bidirectional relationship between P levels and bone can be postulated, in which adequate P availability allows bone mineralization,[1] while osteocytes regulate P levels through FGF23 synthesis and through master control of bone remodeling.[11,12]

Despite this important role of P in bone, it is not known whether serum P is associated with bone mineral density (BMD) or fracture risk at the population level. This research has been scarce and assessed mostly in chronic kidney disease (CKD) patients.[13,14]

The aims of this research were to study the relation between P and BMD and fractures in two population-based cohorts, to study the influence of potential confounders, and to assess the existence of sex-specific effects, which have been previously described for some clinical outcomes mainly in the field of cardiovascular disease.[15,16]

Materials and Methods

This research was performed in three cohorts from the Dutch Rotterdam Study[17] (RS-I, recruitment period 1989–1993, original *n* = 7983; RS-II, recruitment period 2000–2001, original *n* = 3011; RS-III, recruitment period 2006–2008, original *n* = 3932; all subjects aged 45 years or older) and in the US Osteoporotic Fractures in Men (MrOS) study[18,19] (recruitment period 2000–2002, original *n* = 5994; all male subjects aged 65 years or older). Fasting serum P levels were measured in the third follow-up visit of RS-I and in baseline visits of RS-II, RS-III, and MrOS (Fig. 1). Fasting P levels were chosen because the fasting state might modify the association of P with clinical outcomes.[20] Fracture incidence was collected prospectively until January 1, 2007, in RS-I and RS-II, and until January 8, 2015, in MrOS. Fracture incidence was not assessed in RS-III. A total of 12,216 and 11,196 participants were included for the BMD and fracture analyses, respectively, all with signed informed consent. The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus Medical Center; MrOS was approved by the Institutional Review Board of each of the six clinical centers that enrolled participants.

Laboratory measurements

The Rotterdam Study

The concentration of phosphorus in serum corresponds to the inorganic fraction, or *phosphate* (P), based on the formation of ammonium phosphomolybdate.[1] Total calcium (Ca) determination was performed through a colorimetric o-cresolphthalein complexone method. Levels of 25-hydroxyvitamin D (25OHD) were determined through an electrochemiluminescence-based immunoassay (Elecsys Vitamin D Total, Roche Diagnostics, Mannheim, Germany); the test sensitivity was 10 nmol/L, the test range was 7.5 nmol/L to 175 nmol/L, the within-run

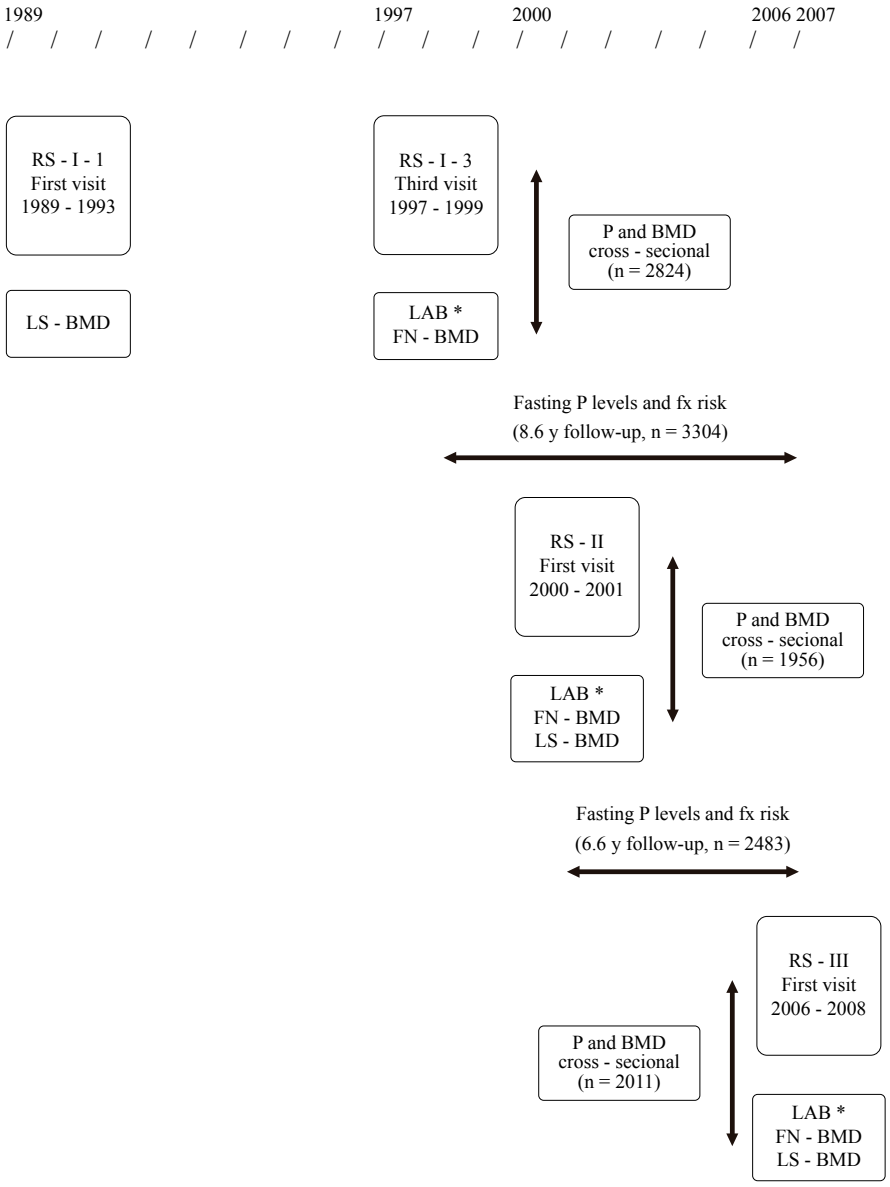


Figure 1. Flowchart for time line, design, and sample sizes for the analyses of the Rotterdam Study cohorts. LAB* includes fasting phosphate levels. FN-BMD = femoral neck BMD; LS-BMD = lumbar spine BMD; P = fasting phosphate levels; Fx risk = fracture risk.

precision <6.5%, and the total precision <11.5%. We applied cosinor regressions to adjust 25OHD levels for season and year.[21] Creatinine was determined through a sarcosine-based colorimetric assay and standardized against isotope dilution mass spectrometry (ID-MS).

MrOS

Serum P, creatinine, and Ca were measured using a Roche COBAS Integra 800 automated analyzer. P detectable range was 0.3 to 20.0 mg/dL, creatinine was 0.2 to 15.0 mg/dL, and Ca was 0.1 to 20.0 mg/dL. Concentrations of 25OHD2 and 25OHD3 were analyzed by liquid chromatography/tandem mass spectrometry (MS) in a subgroup (n = 2351) and added together to obtain total 25OHD levels using multiple reaction monitoring as previously described.[22] Additionally, free concentrations of 25OHD were measured in a subgroup (n = 541) by ELISA (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium) at Future Diagnostics Solutions (Wijchen, The Netherlands). This measurement was validated by comparison with equilibrium dialysis at 37°C in 15 normal samples, yielding a correlation of 0.83. The lower limit of detection was 1.9 pg/mL and its precision was less than 6%.[23] Serum 25OHD levels were adjusted by season. Measurements were performed at the Mayo Medical Laboratories in Rochester, MN, USA.

Parathyroid hormone (PTH) levels were completed using fasting morning blood samples, and samples were frozen until measurement. Immunoradiometric Assay from Scantibodies (3KG600) at Columbia University was used to measure total intact PTH (pg/mL). Fibroblast growth factor 23 (FGF23) levels were completed at the UC Davis Medical Center by two-site monoclonal antibody ELISA using the millipore method. The lower limit of detection was 3.3 pg/mL. Bone turnover markers were measured in a specialized laboratory (CCBR, Synarc, Lyon, France); type I collagen N-propeptide (PINP, Roche Diagnostics) was measured as marker of bone formation, with intra- and interassay coefficient of variation (CV) of <4.4%. For bone resorption, βC-terminal cross-linked telopeptide of type I collagen (βCTX, Roche Diagnostics) was measured, with intra-and interassays CVs <4.2%.[24]

DXA scanning

Trained radiographic technicians performed BMD measurements using dual-energy X-ray absorptiometry (DXA). RS-I participants were assessed at baseline (lumbar spine-LS-BMD, RS-I-1, 1989–1991) and at the third visit (femoral neck FN-BMD, RS-I, 1997–1999), whereas RS-II and RS-III participants were assessed at both skeletal sites at baseline visits (2000–2001; 2006–2008, respectively), as depicted in Fig. 1. A GE Lunar DPX-L densitometer was used in the assessments of RS-I and RS-II, and a Prodigy total body fan-beam densitometer[25] in RS-III (GE Lunar Corp, Madison, WI, USA). MrOS participants were assessed at both skeletal sites at the baseline visit; each US center used a DXA machine of the same model and manufacturer (QDR 4500, Hologic Inc, Waltham, MA, USA).[18] Machines across all six sites were cross-calibrated.

Fracture assessment

In the Rotterdam Study, information on incident clinical fracture events (of all skeletal sites) was obtained from computerized records of general practitioners (GPs) and hospital registries in the research area (covering 80% of the cohort), which are regularly checked by research physicians who review and code the fracture information according to ICD-10;[26] in addition, research physicians regularly followed participant information in the GP's records outside the research area and made an independent review and encoding of all reported events.[27] All fractures are described by a radiologist, and in case of doubt the actual radiographs were reviewed. Finally, an expert in osteoporosis reviewed all coded events for final classification.[28,29]

Because access to medical specialists in The Netherlands is possible only through the GP, we do not anticipate that a considerable number of fractures could have been treated by orthopedic or traumatology surgeons without previous notification by GP. In The Netherlands, there is a 24-hour general practitioner evening and night center available after regular working hours and the GP is automatically

informed after discharge with a report about the diagnosis. Additionally, insurance companies do not cover expenses from the emergency room when patients have not been referred by the GP. Therefore, a significant underestimation of fractures is not anticipated in RS cohorts.

Incident fracture events were reported by participants in MrOS at 4-month intervals on brief mailed questionnaires.[30] The response rates exceeded 99%. Subsequently, study physicians centrally adjudicated reported fractures from medical records. Incident fractures were confirmed by radiology reports or radiographic images when reports were not available.[31] Only fractures that are confirmed by the adjudication process are included in MrOS data set. Health care service providers sent a film copy or digital image of the X-ray to the Coordinating Center for review and confirmation by a radiologist.

Fracture outcomes

Initially, we tested the association between P and all-fracture incidence; subsequently, we analyzed fractures located at the hip, vertebrae, wrist, humerus, and rib. We also included osteoporotic fractures, defined as fractures at any skeletal site except fingers, toes, skull, and facial fractures.[32]

Covariates

Because of previously reported differences in P levels for men and women,[33] we compared its distribution across sexes in the Rotterdam Study applying *t* tests. We assessed the distribution of potential confounders in subjects with FN-BMD information available across P quintiles, applying age-adjusted tests for trend. We included age, body mass index (BMI), smoking status, FN-BMD, prevalent diabetes mellitus, and levels of total Ca, 25OHD, potassium, creatinine, and estimated glomerular filtration rate (eGFR). Prevalent diabetes mellitus and cardiovascular disease were determined as previously described.[34] Alcohol intake was estimated at baseline through a validated food frequency questionnaire.

The Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine levels[35] and the Modification of Diet in Renal Disease (MDRD) study equation[36] were applied to estimate eGFR (mL/min) in the Rotterdam Study and MrOS, respectively. Phosphate intake information collected at the same visit as fasting P was available in a subgroup from MrOS. This dietary information is from the Block Dietary Systems Food Frequency Questionnaire (FFQ), which was specially designed for the MrOS study as a brief FFQ for older adults, based on the NHANES III dietary recall data and including 69 items.

Statistical analyses

A potential association between P levels and BMD was tested through generalized linear models, allowing Gaussian but also non-normal distributions. BMD in sex-specific standard deviations (SD) was set as the dependent variable, and P levels in mg/dL (1 mg/dL = 0.32 mmol/L) was set as the independent variable, adjusted for age, BMI, and smoking; site and race adjustments were included in MrOS. Betas (β) are expressed per 1 mg/dL increase in P levels. Fitness of different models was compared through the Akaike Information Criteria (AIC);[37] linear models with normal distributions displayed lower AIC values, corresponding to a better fit. The results from these analyses were meta-analyzed. LS-BMD was not measured simultaneously to P assessment in RS-I (Fig. 1).

We explored associations of P levels with fracture risk applying Cox models, testing the proportionality of the hazards through Schoenfeld residuals tests.[38] Results from RS-I, RS-II, and MrOS were pooled through study-level meta-analysis, applying a fixed-effects model because of the small number of studies involved.[39] The analysis time was set at the date of blood draw for fasting P levels. Subjects were followed until the first of the following events happened: first fracture, death, loss to follow-up, or censoring. Hazard ratios (HRs) are expressed per 1 mg/dL increase of P levels or in study-specific quintiles.

Adjustments were made first for a basic model including age, BMI, and

smoking;[40–42] site and race were also included in MrOS (Model I). Analyses in RS cohorts were also adjusted for a dummy variable to account for different DXA machines. We further adjusted the analyses for additional covariates included in a full model (Model II), composed of FN-BMD, calcium, potassium, eGFR, alcohol intake, and prevalent cardiovascular and diabetes mellitus; additionally, this model included season-corrected 25OHD adjustment in the full RS cohorts. We have adjusted for total 25OHD levels in MrOS in a subgroup with this information available.

Because of sex differences in P levels[33,43] and in the association of P with several outcomes,[15,16] we explored relations of P with bone traits in sex-combined and in sex-stratified models in RS cohorts.

Sensitivity analyses

To account for the potential confounding effect of renal impairment in the association between P levels and bone traits, we stratified the fracture analyses at an eGFR threshold of 58 mL/min, the estimated cut-off for P counterregulatory hormones triggering in early kidney disease.[44] In MrOS, subgroup analyses were performed in subjects with laboratory results of total and free 25OHD, PTH, and FGF23. Also in MrOS, we adjusted the fracture analyses for phosphate intake (available in 99.3% of the study population).

In addition, we repeated analyses including only subjects from both cohorts with P levels within normal range (0.81 to 1.45 mmol/L; 2.5 to 4.5 mg/dL).

Primary analyses were performed with subjects with complete information on covariates. The completeness of information on covariates for those participants with available P samples was more than 99% in MrOS (with the exception of subgroup analyses) and approximately 75% in the Rotterdam Study cohorts. Subsequently, missing values in the Rotterdam Study cohorts were imputed via multiple imputation with chained equations, following guidelines for imputation

for the Cox model.

Analyses were performed with SPSS (version 21.0, IBM Corp, Armonk, NY, USA), Stata (version 13, StataCorp LP, College Station, TX, USA), and Comprehensive Meta-Analysis (version 2.0).

Results

The distribution of relevant covariates across quintiles of P is depicted in Tables 1 and 2. P and Ca levels were higher in women than in men in the three RS cohorts ($p < 0.001$). P levels lie within normal range (0.81 to 1.45 mmol/L; 2.5 to 4.5 mg/dL) in the vast majority (~95%) of each study population.

Phosphate is not associated with FN-BMD; it is negatively correlated with LS-BMD in men from Rotterdam Study but not MrOS

Tables 3 and 4 show the relationship between P levels and BMD. We found no association between P and FN-BMD (Table 3) in men (pooled β [95% CI]) (β: –0.04 [–0.08 to 0.01], $p = 0.096$). In women, a negative association was found in the age-only adjusted model (β: –0.15 [–0.22 to –0.08], $p < 0.001$), but it became non-significant after adjustment for BMI.

We found a negative relationship between P levels and LS-BMD (Table 4) in the pooled results from men (β: –0.06 [–0.11 to –0.02], $p = 0.007$), which was driven by men from RS cohorts (β: –0.12 [–0.19 to –0.04], $p = 0.002$) and not significant in men from MrOS (β: –0.03 [–0.09 to 0.03], $p = 0.360$). In women, a negative association was found in the age-adjusted model (pooled β: –0.15 [–0.22 to –0.08], $p < 0.001$), but this became non-significant after adjustment for BMI. Therefore, the significant association between P levels and LS-BMD in sex-combined analysis (β: –0.06 [–0.09 to –0.02], $p = 0.004$) was driven by a significant negative association in men.

Phosphate is associated with all-type fracture risk in men and women

Table 5 shows results from analyses of P levels and fracture risk in RS-I, RS-II, and MrOS after follow-up of 8.6, 6.6, and 10.9 years, respectively. During the follow-up period, a total of 1825 cases of incident fractures were recorded. In the basic model, each 1 mg/dL increase in P levels was significantly associated with an increase in all-type fracture risk in male subjects from the Rotterdam Study and in MrOS and borderline significantly in women. In the full model, the associations were statistically significant in all groups: Results for men were hazard ratio (HR) = 1.52 (1.34 to 1.74), $p<0.001$; results for women were 1.32 (1.04 to 1.67), $p = 0.023$; results for sex and study-combined analyses were HR = 1.47 (1.31 to 1.65), $p <0.001$. In MrOS, further adjustments for season-corrected total 25OHD in the full model yielded similar results: HR = 1.49 (1.17 to 1.90), $p = 0.001$; $n = 2345$). In both cohorts, adjustments for vitamin D (using different methods) did not influence results; furthermore, season adjustment in MrOS did not change results. In the full model, there was no statistical evidence for sex interaction in the association between P and fracture risk in RS cohorts ($p_{\text{heterogeneity}} = 0.258$).

Phosphate in quintiles and fracture risk

Analyses of P in quintiles and fracture risk suggested a dose-effect relation in both RS-I (the RS cohort with more fracture events) and MrOS (Tables 6 and 7). After adjustments in Model I, data from RS-I showed a significant trend for increasing P and fracture risk in both men (HRs for the fourth quintile = 2.07 [1.21 to 3.57], $p = 0.008$, and for the fifth quintile = 2.27 (1.33 to 3.90), $p = 0.003$ against the first quintile; $p_{\text{trend}}<0.001$) and women (HRs for the fourth quintile = 1.50 [1.08 to 2.09], $p = 0.016$, and for the fifth quintile = 1.47 [1.05 to 2.05], $p = 0.026$, against the first quintile; $p_{\text{trend}} = 0.022$). A similar trend was observed in MrOS (HRs for the fourth quintile = 1.23 [1.01 to 1.49], $p = 0.040$, and for the fifth quintile: 1.59 [1.32 to 1.93], $p<0.001$, against the first quintile; $p_{\text{trend}}<0.001$).

Table 1. General Characteristics of Subjects With Femoral Neck BMD Information Available in RS-I, RS-II, and RS-III According to Quintiles of Fasting Phosphate Levels

	Men						Women					
	Phosphate in quintiles						Phosphate in quintiles					
	1	2	3	4	5	*p**	1	2	3	4	5	*p**
RS-I												
No.	242	243	243	243	243		322	322	322	322	322	
Phosphate mean (mg/dL)	2.56	2.92	3.14	3.37	3.76		3.02	3.40	3.62	3.84	4.22	
Range (mg/dL)	1.9-2.8	2.8-3.0	3.0-3.3	3.3-3.5	3.5-4.9		2.3-3.3	3.3-3.5	3.5-3.7	3.7-3.9	3.9-5.1	
Age (years)	71.9	72.3	71.7	72.3	71.9	0.982	72.8	72.2	72.9	72.1	72.3	0.297
BMI (kg/m^2)	26.6	26.5	26.4	26.1	26.1	**0.020**	28.7	27.7	27.2	26.6	25.8	**<0.001**
Smoke (%)	87%	87%	93%	92%	94%	**0.002**	47%	49%	52%	52%	48%	0.615
Calcium (mg/dL)	9.58	9.66	9.62	9.64	9.72	**0.001**	9.77	9.79	9.77	9.80	9.86	**0.006**
25OHD (nmol/L)	63.4	61.7	60.5	58.3	59.1	**0.013**	47.2	47.7	45.9	49.7	50.5	**0.057**
FN-BMD (g/cm^2)	0.90	0.90	0.91	0.90	0.88	0.124	0.82	0.80	0.79	0.79	0.78	**<0.001**
Glucose (mmol/L)	6.07	6.01	5.99	5.99	6.16	0.593	6.13	5.83	5.93	5.72	5.76	**0.001**
Prevalent DM (%)	12%	14%	13%	9%	15%	0.623	16%	10%	12%	8%	9%	**0.003**
Creatinine (mg/dL)	1.04	1.05	1.02	1.03	1.06	0.548	0.82	0.82	0.82	0.81	0.82	0.977
eGFR (mL/min)	73.5	72.3	74.7	73.9	73.3	0.676	73.2	73.5	73.5	74.2	73.9	0.632
Na+ (mmol/L)	142.3	142.1	142.4	141.8	142.1	0.218	142.3	142.5	142.7	142.3	142.5	0.957
K+ (mmol/L)	4.32	4.41	4.45	4.43	4.53	**<0.001**	4.30	4.37	4.44	4.43	4.49	**<0.001**

RS-II												
No.	181	182	182	182	182		209	209	210	209	210	
Phosphate mean (mg/dL)	2.48	2.84	3.06	3.29	3.70		2.91	3.31	3.52	3.76	4.14	
Range (mg/dL)	1.4–2.7	2.7–2.9	2.9–3.2	3.2–3.4	3.4–4.7		1.8–3.2	3.2–3.4	3.4–3.6	3.6–3.9	3.9–5.1	
Age (years)	63.4	64.1	64.5	63.5	63.2	0.555	64.2	64.5	63.4	63.8	62.2	**0.002**
BMI (kg/m^2)	27.2	26.7	26.7	26.8	27.1	0.791	28.8	27.7	27.4	26.5	26.1	<**0.001**
Smoke (%)	87%	78%	82%	88%	89%	0.052	57%	63%	60%	57%	63%	0.690
Calcium (mg/dL)	9.52	9.58	9.54	9.57	9.62	**0.014**	9.64	9.65	9.69	9.68	9.74	**0.005**
25OHD (nmol/L)	65.5	68.5	66.3	65.7	63.8	0.355	59.0	56.8	59.6	58.2	63.4	0.294
FN-BMD (g/cm^2)	0.98	0.98	0.95	0.98	0.97	0.478	0.89	0.88	0.91	0.88	0.87	<**0.001**
Glucose (mmol/L)	6.06	5.98	6.17	5.89	6.49	**0.041**	6.13	5.81	5.77	5.83	5.87	0.194
Prevalent DM (%)	12%	9%	15%	11%	20%	**0.024**	13%	8%	10%	10%	9%	0.450
Creatinine (mg/dL)	0.98	0.99	0.99	0.98	0.99	0.571	0.78	0.77	0.79	0.77	0.78	0.640
eGFR (mL/min)	81.8	81.3	80.2	81.9	82.4	0.714	80.8	81.7	80.4	82.3	82.4	0.843
Na+ (mmol/L)	140.9	141.1	141.2	141.1	141.1	0.318	141.2	141.4	141.5	141.6	141.7	**0.032**
K+ (mmol/L)	4.16	4.21	4.21	4.27	4.26	<**0.001**	4.17	4.23	4.24	4.25	4.28	<**0.001**

RS-III												
No.	174	174	174	174	174		228	228	228	228	229	
Phosphate mean (mg/dL)	2.56	2.94	3.20	3.45	3.87		2.97	3.39	3.63	3.85	4.26	
Range (mg/dL)	1.6–2.8	2.8–3.0	3.0–3.3	3.3–3.6	3.6–5.4		2.1–3.2	3.2–3.5	3.5–3.7	3.7–3.9	3.9–5.1	
Age (years)	57.4	57.6	57.4	56.6	55.8	**0.008**	56.2	57.5	57.2	57.6	56.8	0.304
BMI (kg/m^2)	28.2	28.2	27.6	27.4	27.4	**0.017**	29.2	27.9	27.1	27.1	26.9	**<0.001**
Smoke (%)	77%	74%	83%	76%	72%	0.640	64%	67%	69%	60%	69%	0.720
Calcium (mg/dL)	9.68	9.79	9.82	9.87	9.88	**<0.001**	9.74	9.78	9.85	9.90	10.0	**<0.001**
25OHD (nmol/L)	57.5	60.0	59.1	63.1	63.4	**0.011**	56.3	58.1	62.3	59.9	62.3	**0.014**
FN-BMD (g/cm2)	0.98	0.99	0.99	1.00	0.98	0.902	0.93	0.92	0.92	0.91	0.92	0.701
Glucose (mmol/L)	5.92	5.71	5.74	5.78	5.92	0.661	5.40	5.50	5.38	5.41	5.72	0.346
Prevalent DM (%)	12%	8%	10%	12%	14%	0.364	5%	7%	4%	6%	5%	0.764
Creatinin (mg/dL)	0.94	0.97	0.99	0.97	0.97	0.140	0.78	0.77	0.77	0.77	0.78	0.671
eGFR (mL/min)	88.0	85.7	85.9	86.5	88.2	0.391	85.4	86.0	86.2	85.6	85.5	0.664
Na+m (mmol/L)	141.6	141.9	142.1	142.3	142.3	**0.017**	141.8	142.0	142.2	142.0	142.9	**<0.001**
K+ (mmol/L)	4.29	4.39	4.42	4.41	4.45	**<0.001**	4.30	4.33	4.38	4.38	4.44	**<0.001**

BMI = body mass index; smoke = ever smoke; 25OHD = 25-hydroxyvitamin D levels; FN-BMD = femoral neck bone mineral density; prevalent DM = prevalent diabetes mellitus; eGFR = estimated glomerular filtration rate according to Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine levels.

Conversion to SI units: To convert 25-hydroxyvitamin D levels to ng/mL, multiply by 0.4; to convert glucose to mg/dL, multiply by 18.02.

*p** values correspond to age-adjusted significance of trend across quintiles.

Table 2. General Characteristics of Subjects With Femoral Neck BMD Information Available in Men From MrOS According to Quintiles of Fasting Phosphate Levels

	Men					
	Phosphate in quintiles					
	1	2	3	4	5	*p**
MrOS						
No.	1086	1085	1085	1085	1084	
Phosphate mean (mg/dL)	2.6	2.9	3.2	3.4	3.8	
Range	1.8–2.8	2.8–3.0	3.1–3.3	3.3–3.5	3.5–6.8	
Age (years)	73.2	73.2	73.7	73.9	73.7	**0.002**
BMI (kg/m^2)	27.3	27.3	27.4	27.5	27.6	**0.006**
Smoke (%)	61%	60%	63%	63%	67%	<**0.001**
Calcium (mg/dL)	9.28	9.30	9.31	9.32	9.37	<**0.001**
25OHD (nmol/L)	63.3	65.5	65.4	63.6	62.8	0.534
FN-BMD (g/cm^2)	0.79	0.79	0.79	0.78	0.79	0.723
Glucose (mmol/L)	5.79	5.89	5.80	5.85	6.00	**0.003**
Prevalent DM (%)	7%	10%	10%	11%	18%	<**0.001**
Creatinine (mg/dL)	0.99	0.99	1.02	1.02	1.07	<**0.001**
eGFR (mL/min)	88	89	86	85	82	<**0.001**
Na+ (mmol/L)	141.4	141.3	141.4	141.5	141.4	0.296
K+ (mmol/L)	4.19	4.21	4.25	4.30	4.36	<**0.001**

BMI = body mass index; smoke = ever smoke; 25OHD = 25-hydroxyvitamin D levels; FN-BMD = femoral neck bone mineral density; prevalent DM = prevalent diabetes mellitus; eGFR = estimated glomerular filtration rate according to Modification of Diet in Renal Disease (MDRD) study equation.

*p Values correspond to age-adjusted significance of trend across quintiles.

Table 3. Phosphate Levels and Femoral Neck BMD in RS-I, RS-II, RS-III, and MrOS

	Model I			Model II		
	n	β (95% CI)[a]	*p*	*n*	β (95% CI)[a]	*p*
RS-I	1214	−0.11 (−0.24 to 0.01)	0.084	1204	−0.06 (−0.18 to 0.06)	0.328
Men	1610	**−0.24** (−0.35 to −0.13)	<0.001	1596	−0.05 (−0.16 to 0.05)	0.314
Women						
RS-II						
Men	909	−0.09 (−0.23 to 0.06)	0.232	905	−0.07 (−0.21 to 0.07)	0.311
Women	1047	**−0.19** (−0.32 to −0.05)	0.005	1040	−0.01 (−0.13 to 0.12)	0.916
RS-III						
Men	870	−0.03 (−0.17 to 0.11)	0.692	870	0.01 (−0.12 to 0.15)	0.849
Women	1141	−0.02 (−0.13 to 0.10)	0.762	1140	0.07 (−0.04 to 0.19)	0.196
RS combined[b]						
Men	2993	−0.08 (−0.16 to 0.00)	0.050	2979	−0.04 (−0.12 to 0.03)	0.287
Women	3798	**−0.15** (−0.22 to −0.08)	<0.001	3776	0.01 (−0.06 to 0.07)	0.988
MrOS						
Men	5425	−0.02 (−0.08 to 0.04)	0.458	5422	−0.03 (−0.09 to 0.02)	0.215
Studies combined[b]						
Men	8418	−0.04 (−0.09 to 0.01)	0.079	8401	−0.04 (−0.08 to 0.01)	0.096
Women	3798	**−0.15** (−0.22 to −0.08)	<0.001	3776	0.01 (−0.06 to 0.07)	0.988
Sex combined	12,216	**−0.08** (−0.11 to −0.04)	<0.001	12,177	−0.03 (−0.06 to 0.01)	0.171

Model I: age adjusted. Model II: age, body mass index, and smoking adjusted; additional race and site adjustments in MrOS.

[a] Betas are expressed per 1 mg/dL increase in P levels; BMD is expressed in SD.

[b] Studies were pooled applying a fixed effects model.

Table 4. Phosphate Levels and Lumbar Spine BMD in RS-I, RS-II, RS-III, and MrOS

	Model I			Model II		
	n	β (95% CI)[a]	*p*	*n*	β (95% CI)[a]	*p*
RS-I						
Men	1458	**−0.13** (−0.24 to −0.02)	0.021	1437	−0.10 (−0.21 to 0.01)	0.084
Women	2003	**−0.21** (−0.31 to −0.11)	<0.001	1943	−0.09 (−0.19 to 0.01)	0.084
RS-II						
Men	910	**−0.19** (−0.34 to −0.04)	0.012	906	**−0.18** (−0.32 to −0.03)	0.017
Women	1059	**−0.15** (−0.28 to −0.02)	0.027	1051	−0.02 (−0.15 to 0.11)	0.730
RS-III						
Men	766	−0.12 (−0.27 to 0.03)	0.126	766	−0.09 (−0.24 to 0.06)	0.238
Women	1039	−0.06 (−0.18 to 0.07)	0.374	1038	0.02 (−0.10 to 0.14)	0.772
RS combined[b]						
Men	3134	**−0.14** (−0.22 to −0.07)	<0.001	3109	**−0.12** (−0.19 to −0.04)	0.002
Women	4101	**−0.15** (−0.22 to −0.08)	<0.001	4032	−0.04 (−0.10 to 0.03)	0.247
MrOS						
Men	5390	−0.02 (−0.08 to 0.04)	0.495	5387	−0.03 (−0.09 to 0.03)	0.360
Studies combined[b]						
Men	8524	**−0.07** (−0.11 to −0.02)	0.005	8496	**−0.06** (−0.11 to −0.02)	0.007
Women	4101	**−0.15** (−0.22 to −0.08)	<0.001	4032	−0.04 (−0.10 to 0.03)	0.247
Sex combined	12,625	**−0.10** (−0.13 to −0.06)	<0.001	12,528	**−0.06** (−0.09 to −0.02)	0.004

Model I: age adjusted. Model II: age, body mass index, and smoking adjusted; additional race and site adjustments in MrOS.

[a] Betas are expressed per 1 mg/dL increase in P levels; BMD is expressed in SD.

[b] Studies were pooled applying a fixed effects model.

Table 5. Risk of Incidence of All Types of Fractures as a Function of Phosphate Levels in RS-I, RS-II, and MrOS

	Model I			Model II		
	$n_o$fxs/total *n*	HR[a,b] (95% CI)	*p*	$n_o$fxs/total *n*	HR[a,b] (95% CI)	*p*
RS-I						
Men	152/1476	**1.95** (1.37–2.77)	<0.001	116/1094	**1.74** (1.12–2.69)	0.013
Women	390/1828	**1.33** (1.05–1.69)	0.017	279/1325	**1.48** (1.11–1.97)	0.007
Sex combined	542/3304	**1.50** (1.23–1.83)	<0.001	395/2419	**1.54** (1.21–1.95)	<0.001
RS-II						
Men	75/1127	1.33 (0.78–2.25)	0.292	51/876	1.58 (0.84–2.95)	0.153
Women	162/1356	0.90 (0.62–1.31)	0.583	116/1012	1.02 (0.66–1.56)	0.937
Sex combined	237/2483	1.03 (0.76–1.40)	0.829	167/1888	1.18 (0.83–1.69)	0.351
RS combined[c]						
Men	227/2603	**1.73** (1.29–2.32)	<0.001	167/1970	**1.69** (1.18–2.41)	0.004
Women	552/3184	1.19 (0.97–1.45)	0.092	395/2337	**1.32** (1.04–1.67)	0.023
MrOS						
Men	1046/5409	**1.54** (1.34–1.77)	<0.001	1046/5409	**1.50** (1.30–1.72)	<0.001
Studies combined[c]						
Men	1273/8012	**1.57** (1.39–1.78)	<0.001	1213/7379	**1.52** (1.34–1.74)	<0.001
Women	552/3184	1.19 (0.97–1.45)	0.092	395/2337	**1.32** (1.04–1.67)	0.023
Sex combined	1825/11,196	**1.45** (1.31–1.62)	<0.001	1608/9716	**1.47** (1.31–1.65)	<0.001

$n_o$fxs = number of fractures; HR = hazard ratio. Model I: age, body mass index (BMI), and smoking adjusted; additional race and site adjustments in MrOS. Model II: adjusted for age, BMI, smoking, FN-BMD, alcohol intake, prevalent diabetes mellitus, prevalent cardiovascular disease, eGFR, and serum levels of potassium and calcium; additional adjustments for season-adjusted 25(OH)D in RS cohorts and race and site adjustments in MrOS.
[a] Hazard ratios are expressed per increase in 1 mg/dL of P levels. [b] HRs from Cox models.
[c] Studies were combined applying a fixed effects model.

Table 6. Risk of Incidence of All Types of Fractures as a Function of Phosphate Levels Categorized in Quintiles in Men and Women From RS-I

	Men				Women		
P levels[a] mean (range)	Events/no. risk	HR[b,c] (95% CI)	*p*	P levels[a] mean (range)	Events/no. risk	HR[b,c] (95% CI)	*p*
2.6 (1.9–2.8)	20/295	1.00 (reference)		3.0 (2.3–3.3)	59/365	1.00 (reference)	
2.9 (2.8–3.0)	22/295	1.12 (0.61–2.06)	0.708	3.4 (3.3–3.5)	78/366	1.33 (0.95–1.87)	0.099
3.1 (3.1–3.2)	32/295	1.66 (0.95–2.91)	0.075	3.6 (3.5–3.7)	76/365	1.25 (0.89–1.76)	0.197
3.4 (3.3–3.5)	38/295	**2.07** (1.21–3.57)	0.008	3.8 (3.7–3.9)	90/366	**1.50** (1.08–2.09)	0.016
3.8 (3.5–7.6)	40/296	**2.27** (1.33–3.90)	0.003	4.2 (4.0–5.2)	87/366	**1.47** (1.05–2.05)	0.026
		p_{trend} **<0.001**				p_{trend} = **0.022**	

[a] P levels expressed in mg/dL.

[b] Hazard ratios are age, body mass index, and smoking adjusted; first quintile was set as reference.

[c] Hazard ratios are derived from Cox models.

Subtypes of fractures

Results of different subtypes of fractures can be found in Supplemental Table S1. In studies and sexes combined we found that P levels were related to all types of fractures.

Although effects sizes could not be compared due to the difference in numbers of fractures, it appeared that the strongest associations were found for (clinical) vertebral fractures in men while women displayed a stronger association for humerus fractures.

Sensitivity analyses

The stratified fracture analyses according to eGFR (Supplemental Table S2) showed that the association between P and fractures was not abolished after restricting the analyses to subjects with eGFR >58 mL/min (pooled results for sex and studies combined: Model I HR = 1.43 [1.27 to 1.61], $p < 0.001$; Model II HR = 1.44 [1.26 to 1.63], $p < 0.001$).

Additionally, men with eGFR ≤58 mL/min from both populations displayed a significant relation between P and fracture risk in both the basic and full models (RS men, Model I HR = 2.24 [1.01 to 4.98], $p = 0.048$; Model II HR = 4.05 [1.38 to 11.9], $p = 0.011$; men from MrOS, Model I HR = 1.90 [1.40 to 2.58], $p < 0.001$; Model II HR = 1.81 [1.32 to 2.49], $p < 0.001$). The pooled result for men yielded: Model I HR = 1.94 (1.46 to 2.58), $p < 0.001$, and Model II HR = 1.93 (1.42 to 2.62), $p < 0.001$.

Women with eGFR ≤58 mL/min displayed no significant association between P and fracture risk.

Results for P and fracture risk after excluding subjects with abnormal values of P were significant in men (RS men HR = 1.79 [1.26 to 2.56], $p = 0.001$; MrOS men: 1.55 [1.33 to 1.81], $p = 0.001$) (data not shown). The pooled results yielded

HR = 1.59 (1.38 to 1.83), $p < 0.001$. In women, the relation between normal P and fracture risk was not statistically significant (HR = 1.12 [0. 89 to 1.40], $p = 0.330$). In study and sex-combined analyses, the results were HR = 1.44 (1.28 to 1.62), $p < 0.001$.

Analyses after applying multiple imputation did not substantially modify the results obtained in the analyses with the complete cases (data not shown).

Additional adjustments in MrOS

The additional adjustments for total and free 25OHD, FGF23, and PTH levels in a subgroup of men from MrOS (Supplemental Table S3) did not substantially modify the significant association between serum P and fracture risk (PTH-adjusted HR = 1.50 [1.18 to 1.90], $p = 0.001$; FGF23-adjusted HR = 1.69 [1.25 to 2.29], $p = 0.001$; total 25OHD-adjusted HR = 1.49 [1.18 to 1.89], $p = 0.001$; free 25OHD-adjusted HR = 1.73 [1.16 to 2.59], $p = 0.008$). The multivariate analyses showed no modification in the results either.

Table 7. Risk of Incidence of All Types of Fractures as a Function of Phosphate Levels Categorized in Quintiles in Men From MrOS

	Men		
P levels[a] mean (range)	Events/no. risk	HR[b,c] (95% CI)	*p*
2.6 (1.8–2.8)	188/1085	1.00 (reference)	
2.9 (2.8–3.0)	206/1081	1.14 (0.94–1.39)	0.194
3.2 (3.1–3.3)	190/1081	1.06 (0.87–1.30)	0.558
3.4 (3.3–3.5)	213/1083	**1.23** (1.01–1.49)	0.040
3.8 (3.5–6.8)	249/1079	**1.59** (1.32–1.93)	<0.001
		p_{trend} <**0.001**	

[a] P levels expressed in mg/dL.

[b] Hazard ratios are age, body mass index, smoking, site, and race adjusted; first quintile was set as reference.

[c] Hazard ratios are derived from Cox models.

The same pattern was observed after stratification by kidney function (GFR 58 mL/min; Supplemental Table S4) in both strata.

Further adjustments for dietary phosphate intake in men from MrOS did not change results (Model I HR = 1.53 [1.33 to 1.76], *n* = 5394, Model I adjusted for dietary phosphate, calcium, and energy intake HR = 1.53 [1.33 to 1.76], *n* = 5394; Model II HR = 1.48 [1.16 to 1.89] *n* = 2333, Model II adjusted for dietary phosphate, calcium, and energy intake HR = 1.48 [1.16 to 1.89], *n* = 2333).

Additional analyses performed in MrOS in a subset (*n* = 988) with bone turnover markers available did not change results: Model I HR = 1.34 (0.94 to 1.90) *n* = 937, Model I adjusted for PINP and βCTX HR = 1.34 (0.94 to 1.90), *n* = 933; Model II HR = 1.35 (0.94 to 1.95), *n* = 933, Model II adjusted for PINP and βCTX HR = 1.36 (0.94 to 1.97), *n* = 933.

Discussion

In these population-based cohorts, serum P levels were positively and significantly associated with fracture risk in both sexes. These associations were independent of BMD and not explained by multiple potential confounders. Although associations appeared stronger in men than in women in the Rotterdam Study, there was no statistical evidence for a sex difference. P was inversely associated with LS-BMD only in men from the Rotterdam Study, although in combined analyses of sexes and cohorts, this association remained significant. No associations were found with FN-BMD. In women, a relation between P and BMD at both skeletal sites was completely explained by a previously described association of P with BMI.[43]

The results from fracture analyses with P in categories suggested a potential threshold of P (3.3 mg/dL [1.1 mmol/L] in men—consistent in both cohorts—and 3.7 mg/dL [1.2 mmol/L] in women) above which fracture risk was increased. However, trend analysis suggests that risk may start to increase even at lower levels. Analyses restricted to subjects with P levels within normal range still

showed a significant relation between P and fracture risk, although results were statistically significant in men only.

Previous cross-sectional studies reported P levels to be higher in elderly subjects and in CKD patients on hemodialysis with previous fragility fractures[13,14,45] compared with subjects without fractures, but to the best of our knowledge, no prospective studies have been reported at the population level.

Regarding the mechanisms underlying the relation between P levels and bone traits, several potential pathways can be hypothesized, namely: 1) effects through P regulatory hormones; 2) direct effects of P on BMD and or bone quality (and vice versa) and/or fracture risk; and 3) P as a reflection of bone turnover. Regarding the first possibility, P levels are regulated by a complex set of hormones that play an important role in bone metabolism, such as FGF23, PTH, and 1,25-hydroxyvitamin D. Abnormal FGF23 levels have been associated with impaired mineralization,[6,7,46,47] through P-dependent and independent effects.[48] However, consistent with previous research,[49] adjustments for FGF23 levels did not influence the association of P with fracture risk in men from MrOS.

High PTH levels in hypovitaminosis D may also increase fracture risk.[50] Nevertheless, we found that adjustments for 25OHD in RS cohorts and additionally for total and free 25OHD and PTH levels in a subgroup of men from MrOS did not basically modify the association between P and fracture risk. The exclusion of subjects with CKD yielded similar results both in men and women; therefore, we conclude that our findings are not likely explained by secondary elevations of FGF23 or PTH in CKD or by vitamin D deficiency.

On the other hand, we also observed a strong relation between P and fracture risk in men from RS cohorts with CKD, which was replicated in men from MrOS. These results are consistent with an increased gradient of risk for fracture stemming from the increased P load that patients with CKD display,[51] even without overt hyperphosphatemia. This finding was not abolished or even

attenuated after adjustment for FGF23 and PTH levels in MrOS, suggesting that high P itself and not underlying hormonal disturbances may explain the increased fracture risk in this group. As a potential therapeutic possibility, the feasibility of a multicenter randomized trial testing whether P lowering is able to decrease several clinical outcomes in patients with CKD, including bone pain and fracture risk, is currently being evaluated.[52] In contrast, the association between P and fracture risk in women with CKD was not statistically significant.

Regarding our findings of a negative association between P levels and LS-BMD in the pooled results from men, which was driven by men from RS cohorts, we can only speculate whether this is a chance finding or related to the fact that LS-BMD contains more trabecular than cortical bone. It has been previously described that FGF23 expression differs across human bone tissue[53] and that it tends to cluster in osteocytes near the trabecular periphery and the lacuna-canalicular systems,[54–58] in contrast to the expression pattern of other osteocytes (DMP1 osteocytes), which are diffusely located throughout bone. This observation needs to be tested in other cohorts,[59] and if confirmed, it deserves further research. Because LS-BMD measurements can be affected by degenerative changes,[60] more accurate techniques for trabecular volumetric bone assessment might be desirable as well as novel methods to assess more accurately bone microarchitecture that might be influenced by serum phosphate.[7,8]

It is also possible that P may have direct effects on bone metabolism. P itself exerts key roles in growth plate maturation, secondary ossification center formation, and osteoblast differentiation.[2,4] Moreover, high P diets have been shown to increase bone resorption and development of osteoporosis in senescent mice.[61–63] Studies on rats fed high P showed disturbances in P homeostasis and reduced bone mineralization over short- and long-term periods.[64] Therefore, a direct negative effect of increased P intake on bone is quite well possible. In MrOS, adjustment for dietary phosphate intake did not influence the associations between P and fracture risk, but it is currently difficult to accurately estimate phosphorus intake for example, because phosphorus additives from processed food are often not

labeled on food products.[65] But if a relation can be shown between dietary phosphate intake and (bone) health, this may have implications in light of the increasing use of P additives in our diet.[65]

It is important to emphasize that fracture risk was found to be increased within normal values of serum phosphate, suggesting that for bone health the current upper limit may be too high. It has been shown that high dietary intake of P is related to postprandial elevation of serum P,[66] which may not be reflected as fasting P. Indeed, there was no association between dietary P intake and fasting serum P levels in MrOS. Interestingly, the same threshold above which fracture risk was increased in men from both populations (3.3 mg/dL) was previously related to increased cardiovascular risk.[67] To the best of our knowledge, this is the first report to describe this association in a prospective fashion,[13,14] and it may have important clinical consequences for subjects with and without CKD.

In addition, we cannot exclude the possibility that high serum P is merely a reflection of high bone turnover, although we think this is not very likely because under normal circumstances, P exchange with the skeleton yields a neutral balance and bone turnover abnormalities rarely give rise to clinically relevant disturbances in P homeostasis.[11,68,69] Also, the results from adjustments for bone turnover markers in MrOS suggest that bone turnover does not explain the association between P and fracture risk.

Lastly, we cannot rule out that P levels were associated with fracture risk through non-skeletal effects, but an effect through falling appears to be unlikely because P levels were also strongly related to vertebral fractures and these fractures are often not preceded by a fall. Nevertheless, we cannot discard that other potential mechanisms through muscle mass or function might play a role in our findings.

Although there was no statistical evidence for an interaction by sex in the main analysis, the relations between P and bone traits seemed stronger in men than in women with larger effect sizes. Such an observation has been reported before for

other clinical outcomes, such as all-cause mortality, subclinical atherosclerosis, CKD progression, and incident coronary disease.[15,16,70] More research is needed to fully elucidate if the relation of P and fractures (and other outcomes) is indeed stronger in men.

Our study has some limitations. LS-BMD and fasting P levels were not assessed simultaneously at RS-I and only 75% of individuals from RS cohorts had complete data for all covariates of interest. But results were similar after applying multiple imputation. There are several strengths, though, namely the availability of several well-characterized and large cohorts with BMD and prospective fracture information and the ability to replicate the association between P and fracture risk in another large population-based cohort. Although no DXA cross-calibration between MrOS and RS cohorts was performed, statistical adjustments to account for use of different machines did not materially change results.

Additionally, the association between P and fracture risk was significant despite multiple adjustments, including levels of FGF23 and PTH in men from MrOS. Therefore, we consider it unlikely that our results are explained by residual confounding.

In conclusion, we found that in two population-based studies, increasing P levels were positively associated with fracture risk in men and women. These results were independent from BMD, although there was an inverse association between P and LS-BMD in men. The association between P and fractures was also independent of dietary phosphate intake. Results were also not explained by serum levels of calcium, 25OHD, PTH, FGF23, or by comorbidity, including CKD, but associations between P and fractures were also found in men with CKD. The association between P and fractures calls for future research testing whether lowering of serum P or reducing P intake may reduce the risk of fracture. Additionally, further well-powered studies are needed to clarify if there is a sex difference in the relation between P and bone traits.

The findings of our study also suggest that the current upper limit of serum P may be too high and they call for more research into the effects of high P diets and the use of P as food additives on bone health.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

The authors thank the participants and staff of the research center of the Rotterdam Study and the MrOS study. The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam; Netherlands Organization for the Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Ministry of Education, Culture, and Science; the Ministry for Health, Welfare, and Sports; the European Commission (DG XII); and the Municipality of Rotterdam. The Osteoporotic Fractures in Men (MrOS) Study is supported by National Institutes of Health funding. The following institutes provide support: the National Institute of Arthritis and Musculoskeletal and Skin Diseases; the National Institute on Aging; the National Center for Research Resources; and NIH Roadmap for Medical Research under the following grant numbers: U01 AR45580, U01 AR45614, U01 AR45632, U01 AR45647, U01 AR45654, U01 AR45583, U01 AG18197, U01 AG027810, and UL1 TR000128. CMN is supported by NIAMS K01 AR062655. The funding sources had no influence in the study design, collection, analysis, interpretation of data, writing of the report, and in the decision to submit.

Authors' roles: Study design: NCO, NK, and MCZ. Data collection: AH, AU, MCZ, and CMN. Data analysis: NCO, ERH, CMN, and MCZ. Drafting of the manuscript: NCO, NK, CMN, ERH, and MCZ. Critical review of the manuscript: all authors. Statistical analyses: NCO and ERH. Approving final version of manuscript: NCO, NK, CMN, ERH, and MCZ. Obtained funding: AH and AU. Study supervision: MCZ. CMN and ERH had full access to all of the data in the MrOS study; NCO and MCZ had full access to all of the data in Rotterdam Study cohorts. NCO and MCZ take responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. **Berner YN, Shike M.** Consequences of phosphate imbalance. *Annu Rev Nutr* 1988;8:121–48.

2. **Koeppen BM, Stanton BA**. The renal system. In: Koeppen BM, Stanton BA, editors. *Berne & Levy physiology* 6th ed. Philadelphia: Mosby; 2010. p. 557–636.

3. **Hu MC, Shiizaki K, Kuro-o M, Moe OW**. Fibroblast growth factor 23 and klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* 2013;75:503–33.

4. **Zhang R, Lu Y, Ye L, et al**. Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J Bone Miner Res* 2011;26(5):1047–56.

5. **Albright F, Butler AM, Bloomberg E**. Rickets resistant to vitamin D therapy. *Am J Dis Child* 1937;54(3):529–47.

6. **Pettifor JM, Thandrayen K**. Hypophosphatemic rickets: unraveling the role of FGF23. *Calcif Tissue Int* 2012;91(5):297–306.

7. **Shimada T, Kakitani M, Yamazaki Y, et al**. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004;113(4):561–8.

8. **Ichikawa S, Imel EA, Kreiter ML, et al**. A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Clin Invest* 2007;117(9):2684–91.

9. **Masi L, Gozzini A, Franchi A, et al**. A novel recessive mutation of fibroblast growth factor-23 in tumoral calcinosis. *J Bone Joint Surg Am* 2009;91(5):1190–8.

10. **Urakawa I, Yamazaki Y, Shimada T, et al**. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006;444(7120):770–4.

11. **Martin A, David V, Quarles LD**. Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* 2012;92(1):131–55.

12. **Bonewald LF**. The amazing osteocyte. *J Bone Miner Res* 2011;26(2):229–38.

13. **Jadoul M, Albert JM, Akiba T, et al**. Incidence and risk factors for hip

or other bone fractures among hemodialysis patients in the Dialysis Outcomes and Practice Patterns Study. *Kidney Int* 2006;70(7):1358–66.

14. **Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM**. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J Am Soc Nephrol* 2004;15(8):2208–18.

15. **Onufrak SJ, Bellasi A, Cardarelli F, et al**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009;169(1):67–77.

16. **Onufrak SJ, Bellasi A, Shaw LJ, et al**. Phosphorus levels are associated with subclinical atherosclerosis in the general population. *Atherosclerosis* 2008;199(2):424–31.

17. **Hofman A, Brusselle GG, Darwish Murad S, et al**. The Rotterdam Study: 2016 objectives and design update. *Eur J Epidemiol* 2015;30(8):661–708.

18. **Orwoll E, Blank JB, Barrett-Connor E, et al**. Design and baseline characteristics of the osteoporotic fractures in men (MrOS) study—a large observational study of the determinants of fracture in older men. *Contemp Clin Trials* 2005;26(5):569–85.

19. **Blank JB, Cawthon PM, Carrion-Petersen ML, et al**. Overview of recruitment for the Osteoporotic Fractures in men Study (MrOS). *Contemp Clin Trials* 2005;26(5):557–68.

20. **Chang AR, Grams ME**. Serum phosphorus and mortality in the Third National Health and Nutrition Examination Survey (NHANES III): effect modification by fasting. *Am J Kidney Dis* 2014;64(4):567–73.

21. **Robinson-Cohen C, Hoofnagle AN, Ix JH, et al**. Racial differences in the association of serum 25-hydroxyvitamin D concentration with coronary heart disease events. *JAMA* 2013;310(2):179–88.

22. **Singh RJ, Taylor RL, Reddy GS, Grebe SK**. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. *J Clin Endocrinol Metab* 2006;91:3055–61.

23. **Heureux N, Anciaux M, Poncelet M, et al**. Development of an ELISA for the measurement of free 25OHD vitamin D. *Endocrine Abstracts* 2015;37.

24. **Bauer DC, Garnero P, Harrison SL, et al**. Biochemical markers of bone turnover, hip bone loss, and fracture in older men: the MrOS study. *J Bone Miner Res* 2009;24:2032–38.

25. **Hofman A, Breteler MM, van Duijn CM, et al**. The Rotterdam Study: 2010 objectives and design update. *Eur J Epidemiol* 2009;24(9):553–72.

26. World Health Organization. International statistical classification of diseases and related problems. 10th revision (ICD-10). Geneva: WHO; 1992.

27. **de Laet C, van Hout BA, Burger H, et al**. Hip fracture prediction in elderly men and women: validation in the Rotterdam Study. *J Bone Miner Res* 1998;13:1587–93.

28. **Oei L, Zillikens MC, Dehghan A, et al**. High bone mineral density and fracture risk in type 2 diabetes as skeletal complication of inadequate glucose control. *Diabetes Care* 2013;36(6):1619–28.

29. **Stolk L, van Meurs JB, Arp PP, et al**. The RIZ Pro 704 insertion-deletion polymorphism, bone mineral density and fracture risk: the Rotterdam Study. *Bone* 2008;42:286–93.

30. **Barrett-Connor E, Nielson CM, Orwoll E, Bauer DC, Cauley JA, Osteoporotic Fractures in Men Study G**. Epidemiology of rib fractures in older men: Osteoporotic Fractures in Men (MrOS) prospective cohort study. *BMJ* 2010;340:c1069.

31. **Ettinger B, Liu H, Blackwell T, et al**. Validation of FRC, a fracture risk assessment tool, in a cohort of older men: the Osteoporotic Fractures in Men (MrOS) Study. *J Clin Densitom* 2012;15(3):334–42.

32. **Oei L, Hsu YH, Styrkarsdottir U, et al**. A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus. *J Med Genet* 2014;51(2):122–31.

33. **Meng J, Ohlsson C, Laughlin GA, et al**. Associations of estradiol and testosterone with serum phosphorus in older men: the Osteoporotic Fractures in Men study. *Kidney Int* 2010;78(4):415–22.

34. **van Popele NM, Elizabeth Hak A, Mattace-Raso FU, et al**. Impaired fasting glucose is associated with increased arterial stiffness inelderly people without diabetes mellitus: the Rotterdam Study. *J Am Geriatr Soc* 2006;54(3):397–

404.

35. **Levey AS, Stevens LA, Schmid CH, et al**. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150(9):604–12.

36. **Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D**. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999;130(6):461–70.

37. **Akaike H**. An information criterion (AIC). *Math Sci* 1976;14:5–9.

38. **Grambsch PM, Therneau TM**. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika* 1994;81(3):515–26.

39. **Higgins JPT, Greenland S, editors**. Cochrane handbook for systematic reviews of intervention. Version 5.1.0 (updated March 2011). *The Cochrane Collaboration*; 2011.

40. **McCloskey E, Johansson H, Oden A, Kanis JA**. Fracture risk assessment. *Clin Biochem* 2012;45(12):887–93.

41. **Johansson H, Kanis JA, Oden A, et al**. A meta-analysis of the association of fracture risk and body mass index in women. *J Bone Miner Res* 2014;29(1):223–33.

42. **Bleicher K, Cumming RG, Naganathan V, et al**. U-shaped association between serum 25-hydroxyvitamin D and fracture risk in older men: results from the prospective population-based CHAMP study. *J Bone Miner Res* 2014;29(9):2024–31.

43. **Håglin L, Lindblad A, Bygren LO**. Original communication. Hypophosphatemia in the metabolic syndrome. Gender differences in body weight and blood glucose. *Eur J Clin Nutr* 2001;55:493–8.

44. **Isakova T, Wahl P, Vargas GS, et al**. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011;79(12):1370–8.

45. **Figueiredo CP, Rajamannan NM, Lopes JB, et al**. Serum phosphate and hip bone mineral density as additional factors for high vascular calcification scores in a community-dwelling: the Sao Paulo Ageing & Health Study (SPAH). *Bone* 2013;52(1):354–9.

46. **ADHR Consortium**. Autosomal dominant hypophosphatemic rickets is associated with mutations in FGF23. *Nat Genet* 2000;26(3):345–8.

47. **Wang H, Yoshiko Y, Yamamoto R, et al**. Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization in vitro. *J Bone Miner Res* 2008;23(6):939–48.

48. **Sitara D, Kim S, Razzaque MS, et al**. Genetic evidence of serum phosphate-independent functions of FGF-23 on bone. *PLoS Genet* 2008;4(8):e1000154.

49. **Isakova T, Cai X, Lee J, et al**. Associations of FGF23 with change in bone mineral density and fracture risk in older individuals. *J Bone Miner Res* 2016;31(4):742–8.

50. **Bruce DG, St John A, Nicklason F, Goldswain PR**. Secondary hyperparathyroidism in patients from Western Australia with hip fracture: relationship to type of hip fracture, renal function, and vitamin D deficiency. *J Am Geriatr Soc* 1999;47(3):354–9.

51. **Schlieper G, Schurgers L, Brandenburg V, et al**. Vascular calcification in chronic kidney disease: an update. *Nephrol Dial Transplant* 2016;31:31–9.

52. **Bhargava R, Kalra PA, Brenchley P, Hurst H, Hutchison A**. A study to inform the design of a national multicentre randomised controlled trial to evaluate if reducing serum phosphate to normal levels improves clinical outcomes including mortality, cardiovascular events, bone pain, or fracture in patients on dialysis. *Int J Nephrol* 2015;2015:579434.

53. **Mirams M, Robinson BG, Mason RS, Nelson AE**. Bone as a source of FGF23: regulation by phosphate? *Bone* 2004;35(5):1192–9.

54. **Pereira RC, Juppner H, Azucena-Serrano CE, Yadin O, Salusky IB, Wesseling-Perry K**. Patterns of FGF-23, DMP1, and MEPE expression in patients with chronic kidney disease. *Bone* 2009;45(6):1161–8.

55. **Stubbs JR, Liu S, Tang W, et al**. Role of hyperphosphatemia and 1, 25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. *J Am Soc Nephrol* 2007;18(7):2116–24.

56. **Divieti Pajevic P**. Recent progress in osteocyte research. *Endocrinol Metab (Seoul)* 2013;28(4):255–61.

57. **Ubaidus S, Li M, Sultana S, et al**. FGF23 is mainly synthesized by

osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. *J Electron Microsc (Tokyo)* 2009;58(6):381–92.

58. **Wesseling-Perry J, Pereira RC, Wang H, et al**. Relationship between plasma fibroblast growth factor-23 concentration and bone mineralization in children with renal failure on peritoneal dialysis. *J Clin Endocrinol Metab* 2009;94(2):511–7.

59. **Jovanovich A, Buzkova P, Chonchol M, et al**. Fibroblast growth factor 23, bone mineral density, and risk of hip fracture among older adults: the cardiovascular health study. *J Clin Endocrinol Metab* 2013;98(8):3323–31.

60. **Kanis JA, McCloskey EV, Johansson H, Oden A, Melton LJ 3rd, Khaltaev N**. A reference standard for the description of osteoporosis. *Bone* 2008;42(3):467–75.

61. **Koyama Y, Rittling SR, Tsuji K, et al**. Osteopontin deficiency suppresses high phosphate load-induced bone loss via specific modulation of osteoclasts. *Endocrinology* 2006;147(6):3040–9.

62. **Shah BG, Krishnarao GV, Draper HH**. The relationship of Ca and P nutrition during adult life and osteoporosis in aged mice. *J Nutr* 1967;92(1):30–42.

63. **Garcia-Contreras F, Paniagua R, Avila-Diaz M, et al**. Cola beverage consumption induces bone mineralization reduction in ovariectomized rats. *Arch Med Res* 2000;31(4):360–5.

64. **Amato D, Maravilla A, Montoya C, et al**. Acute effects of soft drink intake on calcium and phosphate metabolism in immature and adult rats. *Rev Invest Clin* 1998;50(3):185–9.

65. **European Food Safety Authority**. Assessment of one published review on health risks associated with phosphate additives in food. *EFSA J* 2013;11(11):3444–71.

66. **Nishida Y, Taketani Y, Yamanaka-Okumura H, et al**. Acute effect of oral phosphate loading on serum fibroblast growth factor 23 levels in healthy men. *Kidney Int* 2006;70(12):2141–7.

67. **Dhingra R, Sullivan LM, Fox CS, et al**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community.

Arch Intern Med 2007;167(9):879–85.

68. **Peacock M**. Calcium metabolism in health and disease. *Clin J Am Soc Nephrol* 2010;5 Suppl 1:S23–30.

69. **Coen G, Ballanti P, Mantella D, et al**. Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. *Am J Nephrol* 2009;29(3):145–52.

70. **Bellasi A, Madreoli M, Baldrat L, et al**. Chronic kidney disease progression and outcome according to serum phosphorus in mild-to-moderate kidney dysfunction. *Clin J Am Soc Nephrol* 2011;6(4):883–9.

Supplementary Table 1. Risk of incidence of fractures by fracture location as a function of phosphate levels in RS-I, RS-II and MrOS

	Model I			Model II		
	n	**HR (95% CI)[1,2]**	***p***	**n**	**HR (95% CI) [1,2]**	***p***
Hip fractures						
Men	275/8113	**1.44** (1.09-1.91)	0.010	265/7443	**1.36** (1.02-1.82)	0.037
Women	111/3472	1.06 (0.67-1.67)	0.797	77/2548	1.12 (0.65-1.93)	0.687
Sex-combined	386/11585	**1.32** (1.04-1.68)	0.021	342/9991	**1.30** (1.01-1.68)	0.042
Vertebral fractures						
Men	242/8120	**1.85** (1.39-2.48)	<0.001	219/7447	**1.73** (1.27-2.37)	0.001
Women	167/3467	1.17 (0.81-1.70)	0.405	116/2543	1.16 (0.73-1.83)	0.533
Sex-combined	409/11587	**1.55** (1.24-1.95)	<0.001	335/9990	**1.52** (1.18-1.97)	0.001
Wrist fractures						
Men	112/8111	**1.73** (1.14-2.63)	0.010	104/7439	**1.90** (1.20-2.99)	0.006
Women	151/3400	1.06 (0.72-1.55)	0.772	105/2497	1.18 (0.74-1.89)	0.477
Sex-combined	263/11511	1.33 (1.00-1.76)	0.050	209/9936	**1.51** (1.09-2.09)	0.014
Humerus fractures						
Men	86/8115	**1.65** (1.01-2.70)	0.047	83/6568*	1.61 (0.99-2.62)	0.055
Women	59/3462	**1.96** (1.08-3.56)	0.027	42/2539	**2.16** (1.06-4.40)	0.035
Sex-combined	145/11577	**1.77** (1.21-2.58)	0.003	125/9107	**1.77** (1.18-2.64)	0.005
Rib fractures						
Men	251/8110	**1.35** (1.01-1.80)	0.044	246/7441	**1.40** (1.05-1.88)	0.022
Women	27/3483	0.79 (0.32-1.96)	0.619	21/2556	1.09 (0.37-3.23)	0.873
Sex-combined	278/11593	1.28 (0.98-1.69)	0.074	267/9997	**1.38** (1.04-1.82)	0.026

Osteoporotic fractures[3]						
Men	1223/8050	**1.58** (1.39-1.80)	<0.001	1167/7395	**1.52** (1.33-1.74)	<0.001
Women	525/3237	1.22 (0.99-1.49)	0.060	374/2385	1.28 (1.00-1.64)	0.050
Sex-combined	1748/11287	**1.47** (1.32-1.64)	<0.001	1541/9780	**1.46** (1.30-1.64)	<0.001

Model I: age, BMI and smoking adjusted; additional race and site adjustments in MrOS

Model II: adjusted for age, BMI, smoking, FN-BMD, alcohol intake, prevalent diabetes mellitus, prevalent cardiovascular disease and serum levels of eGFR, potassium and calcium; additional adjustments for season-adjusted 25(OH)D in RS cohorts and race and site adjustments in MrOS

[1] Hazard ratios derive from meta-analysis from RS-I, RS-II and MrOS applying a fixed effects model

[2] HRs from Cox models

[3] Osteoporotic fractures are defined as fractures in any skeletal site except fingers, toes, facial and skull fractures

[*] Pooled result from RS-I and MrOS, due to low number of humerus fracture events (n=1) in men from RS-II (Model II)

Supplementary Table 2. Risk of incidence of all types of fractures as a function of phosphate levels in RS-I, RS-II and MrOS stratified by kidney function

		eGFR>58 cc/min			eGFR≤58 cc/min	
	$n_0$fxs/total n	HR[1,2] (95% CI)	*p*	$n_0$fxs/total n	HR[1,2] (95% CI)	*p*
RS cohorts						
Model I						
Men	198/2349	**1.74** (1.25-2.43)	0.001	29/254	**2.24** (1.01-4.98)	0.048
Women	487/2847	**1.25** (1.01-1.55)	0.037	65/337	0.84 (0.47-1.51)	0.563
Sex-combined	685/5196	**1.39** (1.16-1.66)	<0.001	94/591	1.07 (0.68-1.70)	0.758
Model II						
Men	146/1789	**1.58** (1.07-2.32)	0.020	21/183	**4.05** (1.38-11.9)	0.011
Women	349/2107	**1.37** (1.06-1.77)	0.016	46/235	1.10 (0.55-2.18)	0.790
Sex-combined	495/3896	**1.43** (1.16-1.78)	0.001	67/418	1.39 (0.79-2.44)	0.252
MrOS						
Model I, men	900/4646	**1.48** (1.27-1.73)	<0.001	146/763	**1.90** (1.40-2.58)	<0.001
Model II, men	900/4646	**1.44** (1.23-1.69)	<0.001	146/763	**1.81** (1.32-2.49)	<0.001

Studies combined						
Model I						
Men	1098/6995	**1.52** (1.32-1.75)	<0.001	175/1017	**1.94** (1.46-2.58)	<0.001
Women	487/2847	**1.25** (1.01-1.55)	0.037	65/337	0.84 (0.47-1.51)	0.563
Sex-combined	1585/9842	**1.43** (1.27-1.61)	<0.001	240/1354	**1.65** (1.28-2.13)	<0.001
Model II						
Men	1046/6435	**1.46** (1.26-1.69)	<0.001	167/946	**1.93** (1.42-2.62)	<0.001
Women	349/2107	**1.37** (1.06-1.77)	0.016	46/235	1.10 (0.55-2.18)	0.790
Sex-combined	1395/8542	**1.44** (1.26-1.63)	<0.001	213/1181	**1.76** (1.33-2.33)	<0.001

Model I: age, BMI and smoking adjusted; additional race and site adjustments in MrOS

Model II: adjusted for age, BMI, smoking, FN-BMD, alcohol intake, prevalent diabetes mellitus, prevalent cardiovascular disease and serum levels of potassium and calcium; additional adjustments for season-adjusted 25(OH)D in RS cohorts and race and site adjustments in MrOS

[1] Hazard ratios derive from meta-analysis applying a fixed effects model

[2] HRs from Cox models

Supplementary Table 3a. Additional analyses of the risk of incidence of all-type of fractures as a function of phosphate levels in men from MrOS

		Men	
Model	**n**	**HR**[1,2](95% CI)	***p***
PTH + total 25OHD			
Base	406/2351	**1.49** (1.18-1.89)	0.001
Base + PTH	406/2351	**1.50** (1.18-1.90)	0.001
Base + total 25OHD	406/2351	**1.49** (1.18-1.89)	0.001
Base + PTH + 25OHD	406/2351	**1.50** (1.18-1.90)	0.001
FGF23			
Base	252/1339	**1.69** (1.25-2.29)	0.001
Base + FGF23	252/1339	**1.69** (1.25-2.29)	0.001
PTH + FGF23 + free 25OHD			
Base	146/541	**1.74** (1.16-2.60)	0.007
Base + PTH	146/541	**1.77** (1.19-2.65)	0.005
Base + free 25OHD	146/541	**1.73** (1.16-2.59)	0.008
Base + FGF23	146/541	**1.74** (1.16-2.60)	0.007
Base + PTH + free 25OHD	146/541	**1.77** (1.18-2.64)	0.005
Base + PTH + FGF23	146/541	**1.78** (1.19-2.67)	0.005
Base + free 25OHD + FGF23	146/541	**1.73** (1.15-2.59)	0.008
Base + PTH + free 25OHD + FGF23	146/541	**1.78** (1.19-2.66)	0.005

[1] Models are adjusted for age, BMI, site, smoking and race

[2] Hazard ratios from Cox models

All models are restricted to non-missing variables

Supplementary Table 3b. Additional analyses of the risk of incidence of all-type of fractures as a function of phosphate levels in men from MrOS, stratified by kidney function

	eGFR>58 cc/min			eGFR≤58cc/min		
Model	**n**	**HR**[1,2](95% CI)	*p*	**n**	**HR**[1,2](95%CI)	*p*
PTH + total 25OHD						
Base	347/2004	**1.42** (1.09-1.84)	0.009	59/347	**1.81** (1.02-3.20)	0.043
Base + PTH	347/2004	**1.41** (1.08-1.84)	0.010	59/347	**1.86** (1.05-3.28)	0.033
Base + 25OHD	347/2004	**1.42** (1.09-1.84)	0.008	59/347	**1.81** (1.02-3.21)	0.043
Base + PTH + 25OHD	347/2004	**1.41** (1.08-1.84)	0.011	59/347	**1.85** (1.05-3.27)	0.034
FGF23						
Base	211/1136	**1.70** (1.22-2.37)	0.002	41/203	1.58 (0.74-3.34)	0.234
Base + FGF23	211/1136	**1.70** (1.22-2.37)	0.002	41/203	1.62 (0.76-3.47)	0.211
PTH + FGF23 + free 25OHD						
Base	127/461	**1.80** (1.17-2.79)	0.008	19/80	1.78 (0.50-6.30)	0.373
Base + PTH	127/461	**2.04** (1.30-3.17)	0.002	19/80	1.76 (0.50-6.27)	0.380
Base + 25OHD	127/461	**1.79** (1.16-2.77)	0.008	19/80	1.95 (0.55-7.00)	0.303
Base + FGF23	127/461	**1.81** (1.17-2.79)	0.008	19/80	1.75 (0.48-6.31)	0.393
Base + PTH + 25OHD	127/461	**2.03** (1.30-3.17)	0.002	19/80	1.95 (0.54-7.00)	0.306
Base + PTH + FGF23	127/461	**2.04** (1.31-3.18)	0.002	19/80	1.75 (0.48-6.34)	0.394
Base + free 25OHD + FGF23	127/461	**1.79** (1.16-2.77)	0.008	19/80	1.96 (0.53-7.16)	0.310
Base + PTH + 25OHD + FGF23	127/461	**2.03** (1.30-3.17)	0.002	19/80	1.98 (0.54-7.29)	0.303

[1] Models are adjusted for age, BMI, site, smoking and race

[2] Hazard ratios from Cox models

All models are restricted to non-missing variables

5

Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men

Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men

Authors:

Natalia Campos-Obando, Lies Lahousse, Guy Brusselle,
Bruno H. Stricker, Albert Hofman,
Oscar H. Franco, André G. Uitterlinden, M. Carola Zillikens

Status:

Published in *Eur J Epidemiol* 2018; 33(9):859-71

Abstract

Hyperphosphatemia has been associated with increased mortality in chronic kidney disease but the nature of such a relation in the general population is unclear. To investigate the association between phosphate (P) levels and all-cause and causespecific mortality, we assessed two cohorts from the Rotterdam Study, with follow-up of 14.5 (RS-I) and 10.9 (RS-II) years until January 2012 with availability of fasting phosphate levels. Deaths were classified according to International Classification of Diseases into 7 groups: cardiovascular, cancer, infections, external, dementia, chronic lung diseases and other causes. Sex-stratified Weibull and competing-risks models were adjusted for age, BMI and smoking. Hazard ratios are expressed per 1 mg/dL increase in phosphate levels. The total number of participants included 3731 (RS-I, 2154 women) and 2494 (RS-II, 1361 women) subjects. The main outcome measures were all-cause and cause-specific mortality. A significant positive association was found between phosphate and all-cause mortality in men (pooled HR (95% CI): 1.46 (1.26–1.69)) but not in women (0.90 (0.77–1.05)). In men, higher phosphate increased the risk for cardiovascular mortality (1.66 (1.29–2.14)), other causes (1.67 (1.16–2.40)) and chronic lung disease mortality (1.94 (1.02–3.72)), the latter driven by mortality due to chronic obstructive pulmonary disease (COPD) (4.44 (2.08–9.49)). No relations were found for mortality due to infections, cancer, dementia or external causes. In conclusion, serum P is associated with increased all-cause, cardiovascular and COPD mortality in men but not women. The association with COPD mortality is novel and needs further research on underlying mechanisms.

Introduction

Phosphorus is the sixth most common element in the human body and the second mineral in abundance [1]. It plays an important structural role in hard tissues, such as bone, and exerts critical regulatory roles in metabolic and signaling pathways [1].

The majority of phosphorus is stored in bone (85%) where it is complexed with calcium in the form of hydroxyapatite, whereas 15% of phosphorus is located in the intracellular compartment while less than 1% is present in extracellular fluids. In blood, phosphorus exists in two main forms: a) an organic form bound to proteins (70%), b) an ionized form (30%), known as inorganic phosphorus, or *phosphate*, that circulates freely [1].

Traditionally, phosphate homeostatic mechanisms have been ascribed to the actions of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) [1, 2]. Recently, an equally important new axis of phosphate regulators was discovered [2, 3], composed of the so-called *phosphatonins*: fibroblast growth factor 23 (FGF23), synthesized mainly in osteocytes, and its co-receptor α-Klotho [3, 4]. The FGF23/α-Klotho axis increases P urinary excretion [5].

Monogenic disorders causing extreme phosphate concentrations are associated with rickets in severe hypophosphatemia and calcinosis in severe hyperphosphatemia [5]. Recently, milder hyperphosphatemia was shown to increase cardiovascular mortality in chronic kidney disease (CKD) [6]. Subsequently, this association was reported also in non-CKD population [7–10]. Interestingly, sex differences have been described with associations found in men but not women for all-cause mortality and subclinical atherosclerosis [9]; the underlying reasonsare not understood. In addition to serum phosphate levels (P), high P intake has recently been found to increase mortality [11].

The objectives of this study were to assess the association of P with all-cause

and, in detail, cause-specific mortality within two cohorts of the population-based Rotterdam Study, and to test for potential sex differences in these associations.

Materials and methods

Study population

The Rotterdam Study is a prospective study of men and women designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design has been described elsewhere [12]. This research was performed in two cohorts within the Rotterdam Study, the Rotterdam Study I cohort (RS-I), initiated in 1990 in 7983 subjects, and the Rotterdam Study II cohort (RS-II) initiated in 2000 in 3011 subjects. All participants were 55 years or more at recruitment and have been assessed at baseline and through several follow-up visits. P was measured in the non-fasting state at baseline visit of RS-I (referred to as RS-I-1) and in the fasting state at the second follow-up visit of RS-I (RS-I-3, referred to as RS-I) and the baseline visit of RS-II (Fig. 1). The fasting state may modify the association between P and mortality [10]. Therefore, our main analysis was based on data from RS-I3 and RS-II because P was assessed in the fasting state; subsequently we checked if the observed results followed similar patterns in RS-I-1, where non-fasting samples are available. A total of 3731 participants from RS-I and 2494 from RS-II were included for these analyses, all of them with signed informed consent and available phosphate levels. The Rotterdam Study was approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports. The approval has been renewed every 5 years.

Laboratory measurements

The amount of phosphorus determined in blood corresponds to the inorganic fraction, or *phosphate* (mg/dL), assessed with a method based on the formation of ammonium phosphomolybdate [1].

Total calcium determination (mg/dL) was done through a colorimetric o-cresolphthalein complex one method (Merck Diagnostica, Amsterdam, The Netherlands, for RSI-1; and Roche, Mannheim, Germany, for RS-I and RS-II). Levels of 25-hydroxyvitamin D (nmol/L) were determined through an electrochemiluminescence immunoassay. We applied cosinor regressions to adjust 25-hydroxyvitamin D for season and year. After testing for seasonality applying the dickey fuller test, we proceeded to perform a time transformation on sine and cosine terms (sin(2*pi*time/12)). Afterwards, we proceeded to regress the serum vitamin D levels on those terms to get the mesor, that is, the mean value of the cosinor regression. We then computed the difference between the mean of each season and the mesor, and adjusted every individual value accordingly [13, 14]. Levels of 1,25-dihydroxyvitamin D_3 were assessed in a subset of participants from RS-I-1 through ^{125}I-radioimmunoassay (IDS, Boldon, UK). Creatinine was determined through a sarcosine-based colorimetric assay and standardized against isotope dilution mass spectrometry (ID-MS). Cystatin C was assessed through particle enhanced immunoturbidimetric assay. C-reactive protein (CRP) levels were measured through an agglutination method with antibodies. Magnesium (Mg) levels were determined with a colorimetric method based on xylidyl blue. Glucose and cholesterol levels were determined by standard enzymatic methods [12].

Covariates

We assessed the distribution of potential confounders across P quintiles, such as age, body mass index (BMI), smoking status, calcium, 25-hydroxyvitamin D levels, creatinine, estimated glomerular filtration rate (eGFR), C-reactive protein (CRP), glucose, magnesium, total cholesterol to HDL cholesterol ratio and prevalent diabetes mellitus and cardiovascular disease (CVD). BMI, smoking status, prevalent diabetes mellitus and prevalent CVD were assessed as previously described [12]. The diagnoses of prevalent and incident chronic obstructive pulmonary disease (COPD) cases was based on an obstructive pre-bronchodilator spirometry ($FEV_1/FVC<0.7$), according to GOLD guidelines [15].

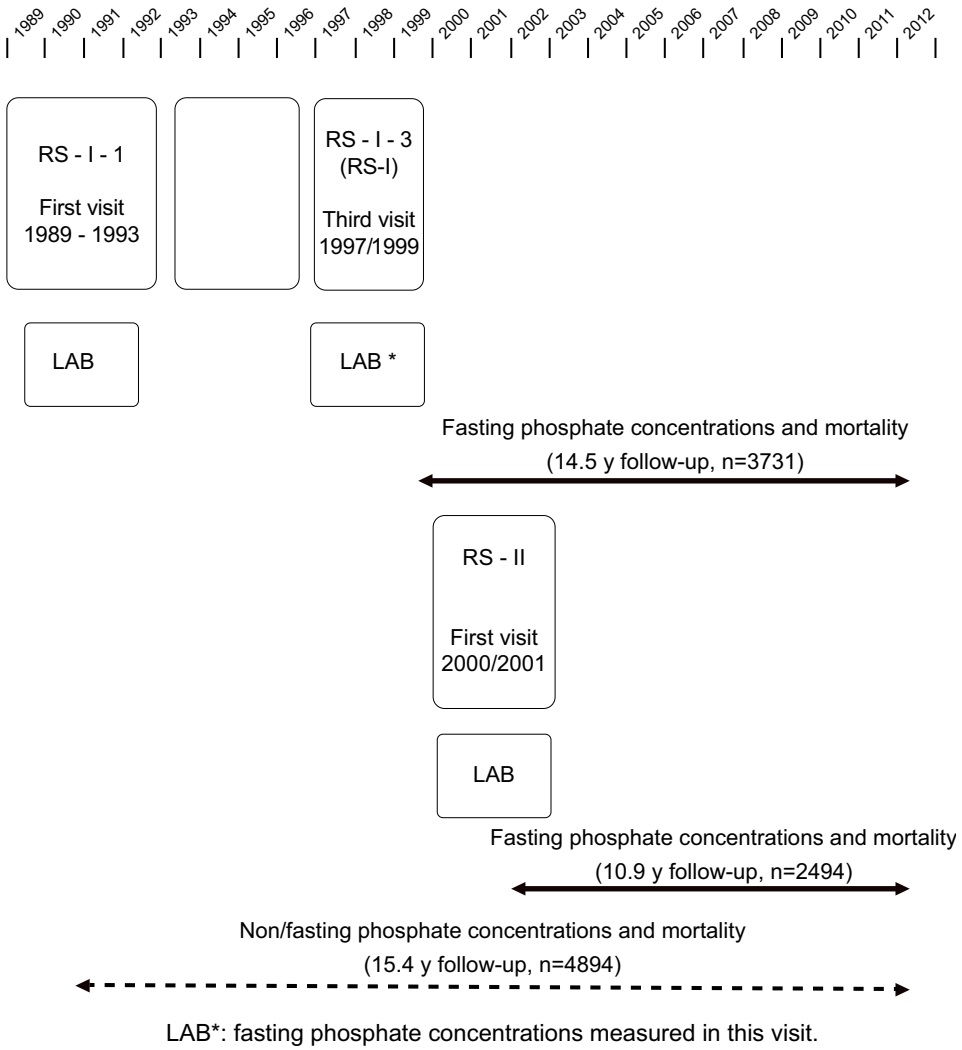


Figure 1. Flowchart for time line, design and sample size for the analyses

P intake at baseline visit (RS-I-1) was collected in a subsample of participants through a validated semiquantitative food frequency questionnaire. The Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine [16, 17] were applied to estimate eGFR (mL/min). Additionally, cystatin C-based eGFR was estimated for subjects with creatinine-based eGFR less than 60 mL/min, as previously recommended [16].

Assessment of all-cause and cause-specific mortality

Information on vital status is obtained continuously from the municipal authorities in Rotterdam. The cohorts are monitored for mortality through computerized linkage of the study database to medical files of general practitioners. Two research physicians independently coded the mortality events according to ICD-10. Medical specialists in the respective field reviewed and confirmed the diagnosis. Information on cause-specific mortality was available until January, 2012.

Different causes of mortality were recorded according to ICD-10 codes and firstly grouped into cardiovascular diseases (CVD), cancer and other causes. To perform comprehensive analyses, the group of other causes was further categorized into external causes, dementia, infections, chronic lung disease and other causes in the strict sense, as previously described [18].

Statistical analysis

Subjects with fasting P measurements from RS-I and RS-II were analyzed separately and in a meta-analysis. Additionally, we analyzed subjects with non-fasting P from RSI-1.

Due to sex differences in P [19] and in its association with health outcomes [9], we built sex-stratified analyses.

We compared the distribution of potential confounding factors applying age-adjusted tests of trend across P quintiles. We estimated P levels across smoking categories applying ANOVA and post hoc (Tukey's) tests. Initially, the association of P with mortality was assessed through Cox models, testing the proportionality assumption of the hazards via the Schoenfeld residuals test. All significant HRs from Cox' models were found to be constant over follow-up time; therefore,

we found no evidence for a time-dependent effect of P levels on mortality. In a second step, we compared the semi-parametric Cox model with parametric models, and found that Weibull regression models—albeit with highly similar results to Cox regressions— provide better statistical fit to the data than Cox models. Weibull models provided also better fit than the rest of parametric models. We applied Cox-Snell residuals graphs and Akaike (AIC) and Bayesian information criteria (BIC) to compare among models, as previously recommended [20]. Models with lower AIC and BIC correspond to a better fit. Therefore, the results reported in this manuscript correspond to Weibull regression models. Finally, we also performed competing-risks regressions models which allow for informative censoring due to the multiple possible causes of death [21]; these models provide an estimate of the effect of the exposure on the probability of developing the outcome over time [22].

Hazard ratios (HRs) are expressed per increase in 1 mg/ dL (0.32 mmol/L) of P or in quintiles; the latter were built to explore a potential dose–effect relationship between phosphate levels and mortality.

The analysis time was set at the date of blood drawing. Subjects were followed until the first of the following events happened: death, lost to follow-up, or censoring by 1st January, 2012.

Adjustments were made firstly for age, BMI and smoking because they are related to mortality and P; subsequently other covariates that have been associated with mortality were added to the model and retained if they changed the beta estimate more than 10%, including eGFR, glucose, hsCRP, Mg levels, cholesterol to HDL cholesterol ratio, calcium, 25-hydroxyvitamin D and prevalent cardiovascular disease.

Results from RS-I and RS-II were meta-analyzed using fixed-effect model.

Primary analyses were done with subjects with complete information on

covariates. Subsequently, missing values were imputed via multiple imputation with chained equations, allowing missingness at random. We followed specific guidelines for imputation for survival analysis.

Sensitivity analyses

We repeated analyses including only subjects with normal P (2.5–4.5 mg/dL; 0.81–1.45 mmol/L). We further adjusted the analyses for phosphate dietary intake and 1,25- dihydroxyvitamin D_3 levels in a subset of participants from RS-I-1 (n = 4046).

Additionally, we performed stratified analyses according to smoking categories.

We used SPSS (version 21.0, Armonk, NY: IBM Corp), Stata (version 13, College Station TX: Stata Corp LP) and Comprehensive Meta-Analysis (version 2.2, Biostat, Englewood, NJ). A two-sided $p<0.05$ was considered significant.

Results

Serum phosphate correlates

A general descriptive summary of main continuous covariates is depicted in Table 1. The distribution of relevant covariates and risk factors across even quintiles of P for RS-I and RS-II is depicted in Table 2. P was higher in women than men in both cohorts ($p_{\text{difference}}<0.001$). P levels were different across smoking categories in both sexes and cohorts (ANOVA $p<0.001$); this difference was due to higher P in current smokers (Tukey's tests >0.05 between former and never smokers).

P was within normal range in 95.5 and 94.9% of participants in the fasting state (RS-I and RS-II, respectively) and in 89.7% of participants in the non-fasting state (RS-I-1).

Serum phosphate and all-cause mortality

During 14.5 year (median) and 10.9 year (mean) follow-up a total of 1631 and 469 fatal events occurred in RS-I and RS-II, respectively. We found a significant interaction between P and sex for all-cause mortality in RS-I ($p_{interaction}$<0.001) and performed sex-stratified analyses. The results for the comparison of goodness-of-fit between parametric models and the semiparametric Cox model are displayed in Supplementary Table 1 (AIC and BIC criteria) and in Fig. 2 (Cox-Snell residuals plot). Both methods showed that Weibull models provide a better fit to our data among the parametric and semiparametric models.

The associations between P and all-cause mortality are depicted in Table 3. Results from RS-I and RS-II were meta-analyzed (pooled HR (95% CI)). A significant association between P and all-cause mortality was found in men (1.46 (1.26–1.69)) but not in women (0.90 (0.77–1.05)).

Table 1. General characteristics of subjects in RS-I and RS-II with serum phosphate levels, BMI and smoking information available, stratified by sex.

	Men			Women		
	Mean (SD) (n: 1577)	Min.	Max.	Mean (SD) (n: 2154)	Min.	Max.
(I) RS-I						
Age (year)	71.8 (6.53)	61.4	96.7	72.5 (7.06)	61.4	100.9
BMI (kg/m^2)	26.3 (3.18)	17.6	41.1	27.3 (4.37)	15.2	47.9
Calcium (mg/dL)	9.65 (0.39)	6.26	11.6	9.79 (0.41)	6.98	12.9
Phosphate (mg/dL)	3.15 (0.44)	1.91	7.62	3.62 (0.43)	2.28	5.25
25(OH)D (nmol/L)	61.4 (25.5)	8.99	173.8	47.9 (22.5)	5.14	134.4
CRP (mg/L)	4.24 (7.22)	0.20	115.0	3.93 (6.66)	0.20	145.0
Glucose (mmol/L)	6.06 (1.62)	4.10	20.5	5.87 (1.46)	1.60	19.5
Creatinine (μmol/L)	92.4 (33.6)	43.0	1107.0	72.1 (14.8)	34.0	263.0
eGFR (mL/min)	73.8 (14.4)	3.55	108.8	73.8 (13.9)	14.9	113.7
Mg (mmol/L)	0.85 (0.06)	0.60	1.13	0.85 (0.06)	0.58	1.17
Chol to HDL ratio	4.69 (1.32)	1.52	10.2	4.30 (1.30)	1.19	14.1

	Men (n: 1133)			Women (n: 1361)		
(II) RS-II						
Age (year)	64.3 (7.48)	55.1	93.9	64.9 (8.17)	55.1	95.3
BMI (kg/m^2)	26.9 (3.36)	16.8	40.5	27.4 (4.46)	15.9	50.5
Calcium (mg/dL)	9.57 (0.34)	8.58	11.8	9.68 (0.34)	8.70	11.3
Phosphate (mg/dL)	3.09 (0.44)	1.39	4.66	3.54 (0.44)	1.82	5.12
25(OH)D (nmol/L)	65.7 (27.9)	0.25	175.0	58.9 (27.5)	5.84	162.5
CRP (mg/L)	2.37 (4.60)	0.30	51.8	2.33 (4.16)	0.00	65.5
Glucose (mmol/L)	6.17 (1.78)	3.90	22.1	5.87 (1.47)	3.80	25.9
Creatinine (μmol/L)	87.8 (18.7)	53.0	349.0	69.2 (11.8)	40.0	165.0
eGFR (mL/min)	80.9 (13.9)	14.0	111.6	80.6 (13.7)	26.8	108.4
Mg (mmol/L)	0.83 (0.06)	0.34	1.02	0.83 (0.06)	0.43	1.06
Chol to HDL ratio	4.77 (1.34)	1.83	12.4	4.23 (1.22)	1.52	11.1

BMI body mass index, 25*(OH)*D 25-hydroxyvitamin D levels, *CRP* C-reactive protein, *eGFR* estimated glomerular filtration rate, *Mg* magnesium, *Chol to HDL ratio* total cholesterol to HDL cholesterol ratio

Conversion to SI Units: to convert 25-hydroxyvitamin D levels to ng/mL multiply by 0.4; to convert glucose to mg/dL multiply by 18.02; to convert creatinine to mg/dL multiply by 0.011; to convert magnesium to mg/dL multiply by 2.43

Min: minumum. Max: maximum

Adjustments in a full model composed of age, BMI, smoking, prevalent cardiovascular disease and levels of calcium, 25-hydroxyvitamin D, eGFR, CRP, Mg, glucose and total cholesterol to HDL cholesterol ratio levels did not substantially modify results (men: 1.49 (1.27–1.74); women: 0.92 (0.79–1.07)).

Similarly, results from RS-I-1 with non-fasting phosphate showed a significant association of phosphate with all-cause mortality in men (1.12 (1.02–1.23); n_o events = 1389), but not in women (0.99 (0.91–1.08); n_o events = 1779).

To explore whether there was a dose–response pattern in the association we found in men, we analyzed P in even quintiles and all-cause mortality in RS-I, the cohort with most events, (Table 4) and set the first quintile (lowest) as reference. We observed a significant trend for increasing P and mortality (p_{trend}<0.001) with significant HRs for the fourth (1.35 (1.08–1.69)) and fifth quintile (1.49 (1.19–1.86))

compared with the first quintile.

Sensitivity analyses

Results after excluding subjects with abnormal P were similar to the unrestricted analyses (men: 1.44 (1.21–1.70); women: 0.87 (0.74–1.03)). Adjustments for phosphate and energy intake in men from RS-I-1 did not modify the results between non-fasting phosphate and all-cause mortality (1.13 (1.02–1.24); n_o events = 1117). Further adjustments for 1,25 dihydroxyvitamin D_3 levels in a subset from RS-I-1 did not modify results (data not shown).

Serum phosphate and cause-specific mortality in men

We did not observe associations between P and cause-specific mortality in women (data not shown). In contrast, the pooled results in men (Table 5) showed a significant positive relation between P and CVD mortality (1.66 (1.29–2.14)). Exclusion of male subjects with prevalent CVD disease yielded similar results (1.69 (1.28–2.23)).

We also found an association between higher P and chronic lung disease mortality (1.94 (1.02–3.72)). Most of these cases clustered within COPD mortality. Therefore, we further investigated such a relation (Table 6), and found a significant association (4.44 (2.08–9.49)). Most likely due to power constraints, this association was not significant in RS-II (05 cases in contrast to 28 cases in RS-I) but there was no evidence for statistical difference between both estimates ($p_{\text{heterogeneity}} = 0.780$).

Table 2. General characteristics of subjects in RS-I and RS-II according to quintiles of fasting phosphate levels

	Men						Women					
	Phosphate in quintiles						Phosphate in quintiles					
	1	2	3	4	5	*p**	1	2	3	4	5	*p**
(I) RS-I												
N (mg/dL)	315 (2.56)	315 (2.92)	316 (3.15)	315 (3.37)	316 (3.77)		431 (3.02)	431 (3.40)	431 (3.62)	431 (3.83)	430 (4.21)	
Age (year)	71.6	72.4	71.4	71.9	71.8	0.968	73.0	72.3	72.8	72.4	71.9	**0.049**
BMI (kg/m^2)	26.7	26.4	26.2	26.2	26.0	**0.005**	29.0	27.5	27.2	26.7	25.9	**< 0.001**
Ever smoke (%)	90%	87%	92%	92%	95%	**0.008**	47%	47%	50%	53%	51%	0.091
Calcium (mg/dL)	9.59	9.66	9.62	9.66	9.72	**< 0.001**	9.77	9.80	9.76	9.79	9.84	**0.026**
25 (OH) D (nmol/L)	62.8	62.6	62.7	59.1	59.9	**0.034**	45.4	48.9	46.8	48.6	50.1	**0.035**
CRP (mg/L)	4.57	3.62	4.15	3.79	5.12	0.340	4.92	4.06	3.79	3.60	3.23	**< 0.001**
Glucose (mmol/L)	6.09	5.96	6.04	6.04	6.15	0.529	6.18	5.79	5.87	5.77	5.77	**< 0.001**
Prevalent DM (%)	14%	12%	13%	14%	15%	0.424	17%	10%	12%	9%	9%	**0.001**

Creatinine (µmol/L)	91.4	92.7	90.2	91.3	96.2	0.167	72.4	72.7	71.5	71.9	71.9	0.652
eGFR (mL/min)	73.9	72.0	75.0	74.2	73.7	0.432	73.1	73.1	74.2	74.1	74.4	0.356
Mg (mmol/L)	0.84	0.84	0.85	0.85	0.86	**0.002**	0.84	0.85	0.85	0.85	0.86	**< 0.001**
Chol to HDL ratio	4.75	4.93	4.75	4.56	4.47	**< 0.001**	4.41	4.42	4.33	4.17	4.18	**< 0.001**
Prevalent CVD (%)	7%	9%	8%	7%	10%	0.221	4%	2%	2%	4%	3%	0.712
(II) RS-II												
N (mg/dL)	226 (2.49)	227 (2.86)	226 (3.07)	227 (3.31)	227 (3.71)		272 (2.92)	272 (3.32)	272 (3.54)	272 (3.77)	273 (4.14)	
Age (year)	63.8	64.4	65.0	64.7	63.8	0.884	65.4	66.2	64.4	65.2	63.1	**< 0.001**
BMI (kg/m^2)	27.1	26.7	26.8	26.7	27.3	0.482	29.1	27.9	27.4	26.8	26.0	**< 0.001**
Ever smoke (%)	86%	81%	80%	87%	89%	0.142	57%	62%	57%	58%	63%	0.642
Calcium (mg/dL)	9.50	9.59	9.53	9.58	9.64	**< 0.001**	9.64	9.66	9.70	9.68	9.75	**0.001**
25 (OH)D (nmol/L)	66.6	68.0	65.1	65.7	62.8	0.103	57.1	57.2	58.3	58.9	63.2	0.071
CRP (mg/L)	2.41	2.22	2.42	1.88	2.93	0.479	2.82	2.54	1.92	2.34	2.01	**0.037**
Glucose (mmol/L)	6.08	5.98	6.24	6.06	6.50	**0.013**	6.10	5.84	5.79	5.78	5.84	**0.049**

Prevalent DM (%)	11%	9%	15%	11%	21%	**0.002**	12%	9%	10%	9%	7%	0.164
Creatinine (µmol/L)	87.6	87.4	88.7	86.8	88.2	0.915	69.5	70.0	68.4	69.2	68.9	0.923
eGFR (mL/min)	80.9	81.3	79.2	81.3	81.7	0.475	79.7	79.1	81.6	80.5	81.8	0.691
Mg (mmol/L)	0.83	0.83	0.83	0.83	0.84	0.290	0.82	0.83	0.83	0.83	0.84	**< 0.001**
Chol to HDL ratio	4.93	4.71	4.62	4.66	4.93	0.906	4.35	4.20	4.31	4.15	4.12	**0.042**
Prevalent CVD (%)	8%	11%	13%	9%	13%	0.351	3%	3%	3%	3%	1%	0.608

Statistically significant *p*-values (<0.05) are highlighted in bold font

**P* values corresponds to age-adjusted significance of trend across quintiles

BMI body mass index, *25(OH)D* 25-hydroxyvitamin D levels, *CRP* C-reactive protein, *prevalent DM* prevalent diabetes mellitus, *eGFR* estimated glomerular filtration rate, *Mg* magnesium, *Chol to HDL ratio* total cholesterol to HDL cholesterol ratio, *prevalent CVD* prevalent cardiovascular disease

Conversion to SI Units: to convert 25-hydroxyvitamin D levels to ng/mL multiply by 0.4; to convert glucose to mg/dL multiply by 18.02; to convert creatinine to mg/dL multiply by 0.011; to convert magnesium to mg/dL multiply by 2.43

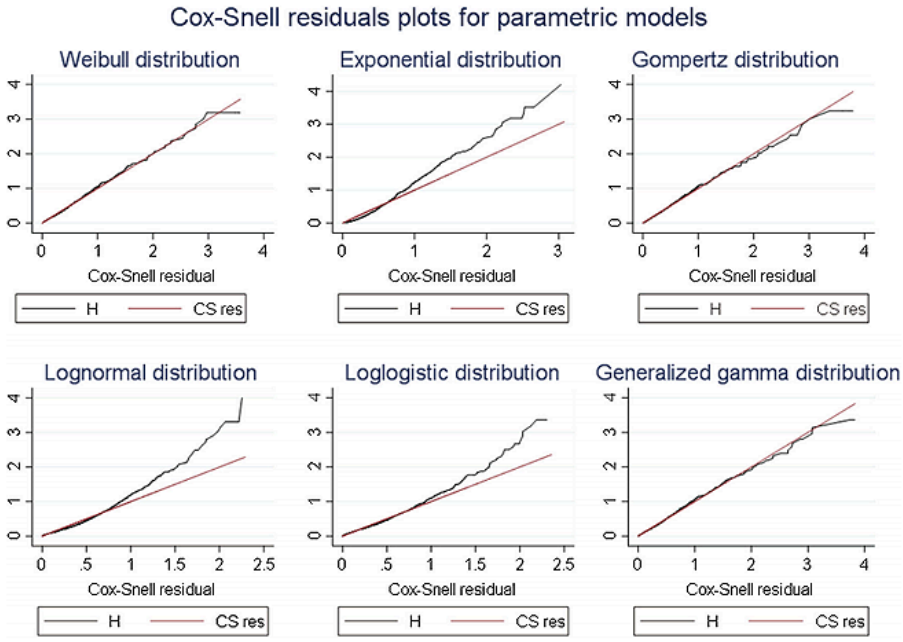


Figure 2. Cox-Snell residuals plot for parametric models in the association between serum phosphate levels and all-cause mortality in men

Table 3. Serum phosphate levels and all-cause mortality in RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012

	Men			Women		
	n_o events	HR* (95% CI)	*p*	n_o events	HR* (95% CI)	*p*
RS-I	810/1577	**1.58** (1.34–1.87)	< 0.001	821/2154	0.85 (0.71–1.00)	0.056
RS-II	262/1133	1.14 (0.85–1.53)	0.378	207/1361	1.14 (0.81–1.60)	0.439
Studies combined†	1072/2710	**1.46** (1.26–1.69)	< 0.001	1028/3515	0.90 (0.77–1.05)	0.176

Statistically significant p-values (<0.05) are highlighted in bold font

*Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels

†Studies combined from meta-analyses using fixed-effect models

Table 4. Serum phosphate levels in quintiles and all-cause mortality in men from RS-I, adjusted for age, BMI and smoking, follow-up until year 2012

Quintile	Phosphate concentrations mean (range)*	n_o events/n_o risk	HR† (95% CI)	*p*
1	2.56 (1.91–2.81)	139/315	1 (reference)	
2	2.92 (2.81–3.02)	154/315	1.09 (0.87–1.38)	0.439
3	3.15 (3.02–3.27)	154/316	1.05 (0.83–1.33)	0.660
4	3.37 (3.27–3.49)	172/315	**1.35** (1.08–1.69)	0.008
5	3.77 (3.52–7.62)	191/316	**1.49** (1.19–1.86)	<0.001
p_{trend}				<0.001

Statistically significant p-values (<0.05) are highlighted in bold font

*Phosphate levels in mg/dL

†Hazard ratios from Weibull models; first quintile was set as reference

Table 5. Serum phosphate levels and cause-specific mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012

	Individual cohorts				Studies combined	
	Cohort	n	HR* (95% CI)	*p*	HR† (95% CI)	*p*
CVD	RS-I	266	**1.80** (1.35–2.39)	<0.001	**1.66** (1.29–2.14)	<0.001
	RS-II	77	1.25 (0.73–2.15)	0.412		
Cancer	RS-I	243	**1.41** (1.04–1.90)	0.025	1.23 (0.95–1.58)	0.112
	RS-II	98	0.88 (0.55–1.40)	0.586		
External	RS-I	18	1.58 (0.50–5.02)	0.439	0.94 (0.36–2.46)	0.902
	RS-II	9	0.29 (0.05–1.62)	0.159		
Infectious	RS-I	56	1.02 (0.53–1.98)	0.943	0.97 (0.53–1.80)	0.929
	RS-II	9	0.71 (0.13–3.84)	0.691		
Dementia	RS-I	52	1.83 (0.93–3.60)	0.081	1.70 (0.92–3.15)	0.092
	RS-II	13	1.18 (0.26–5.37)	0.826		
Lung	RS-I	42	2.07 (0.97–4.42)	0.058	**1.94** (1.02–3.72)	0.044
	RS-II	15	1.64 (0.47–5.72)	0.441		
Other	RS-I	133	**1.58** (1.04–2.41)	0.032	**1.67** (1.16–2.40)	0.006
	RS-II	40	1.98 (0.96–4.11)	0.066		

Statistically significant *p*-values (<0.05) are highlighted in bold font

*Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels

†Studies combined from meta-analyses using fixed-effect models

Further adjustments for glomerular filtration rate did not abolish the association between P and COPD mortality (4.16 (2.05–8.43)). Furthermore, the association was found to be consistent in subjects without chronic kidney disease (CKD) (6.58 (2.59–16.7)); whereas we found no association in subjects with CKD (1.14 (0.20–6.63)), although the latter analysis is constrained due to low number of events and driven only by RS-I. Non-fasting phosphate levels and COPD mortality in men from RS-I-1 also displayed a significant association (1.54 (1.05–2.27), n_o events = 69).

P was also found to be positively associated with mortality from other causes (1.67 (1.16–2.40))

Table 6. Serum phosphate levels and chronic obstructive pulmonary disease (COPD) mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012

	Individual cohorts			Studies combined	
	n	HR* (95% CI)	*p*	HR† (95% CI)	*p*
RS-I	28	**4.62** (2.06–10.3)	< 0.001	**4.44** (2.08–9.49)	< 0.001
RS-II	05	3.29 (0.35–30.7)	0.296		

Statistically significant *p*-values (<0.05) are highlighted in bold font

*Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels

†Studies combined from meta-analyses using fixed-effect models

We found no significant associations between P and death due to cancer, infections, dementia or external causes.

Results from competing-risks regression models were similar to Weibull models and showed a significant association between P and mortality due to CVD (1.50 (1.12–2.02)), other causes (1.40 (1.01–1.93)) and COPD (2.42 (1.62–3.63)); no other significant associations were found (Supplementary Tables 2 and 3).

Analyses after applying multiple imputation yielded significant associations for P and all-cause, CVD, COPD and other causes of mortality in men (data not shown). Missingness of covariates of interest was less than 6%.

Sensitivity analyses

Results after excluding male subjects with abnormal P were similar to the unrestricted analyses (Supplementary Tables 4 and 5). Likewise, our findings remained essentially unaltered after adjustments for calcium and 25-hydroxyvitamin D levels; and were only slightly attenuated after further adjustments for levels of calcium, 25-hydroxyvitamin D and eGFR (CVD 1.65 (1.27–2.14), COPD 3.79 (1.87–7.69), other causes 1.76 (1.21–2.56)). Similar results were obtained after adjustments for cystatin-based eGFR. Additionally, the analyses after exclusion of male subjects with eGFR <60 mL/min showed a positive association between P and mortality due to other causes (1.72 (1.13–2.61)) and COPD (6.58 (2.59–16.7)) - as previously mentioned - and a borderline association between P and CVD mortality (1.36 (1.00–1.85)).

Smoking adjustment did not attenuate the association between P and CVD or COPD mortality (data not shown). The results from the stratified analyses according to smoking categories (Supplementary Tables 6 and 7) showed that in studies combined the associations between P and all-cause and CVD mortality were in the same direction and did not show statistical evidence for a difference across categories ($p_{\text{heterogeneity}} = 0.752$ for all-cause mortality and $p_{\text{heterogeneity}} = 0.796$ for CVD mortality). The relation between P and COPD mortality in men from RS-I (RS-II excluded due to few events) was not statistically different among former and current smokers ($p_{\text{heterogeneity}} = 0.494$).

As previously mentioned, analyses in men from RS-I-1 showed that non-fasting phosphate levels were also associated with chronic lung disease mortality and COPD mortality, and these associations were not abolished after further adjustments for phosphate and energy intake: chronic lung disease mortality: 1.79 (1.19–2.68); n_o events = 59; COPD mortality: 1.87 (1.20–2.91), n_o events = 49.

Discussion

This prospective population-based cohort study among elderly demonstrated that P was positively associated with all-cause mortality in men but not in women, supporting an effect modification by sex previously described [9]. When analyzing in detail cause-specific mortality in men, we found that this association was driven by mortality due to CVD, COPD and other causes. The association between increasing P and the composite endpoint of fatal and nonfatal CVD incidence in non-CKD population in sex-combined analyses has been reported before but is still scarce [7–9]. Our results provide evidence of an association between higher P - even within normal range - and death due to CVD in men. On the other hand, to the best of our knowledge the association we found with COPD mortality is novel. These results remained significant after adjustments for several potential confounders, were observed also after restricting the analyses to subjects with normal P and showed no heterogeneity between cohorts.

Several mechanisms have to be considered when analyzing P and mortality, including phosphate being a marker of another risk factor or through direct pathogenic pathways.

First, P levels are regulated by a complex interplay of factors that have been linked to mortality, such as 1,25-dihydroxyvitamin D_3, PTH and FGF23. Low levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D_3 have been found to be associated with increased mortality [23]. Nevertheless, the vitamin D adjustments did not modify our results.

PTH abnormalities have also been associated with mortality. Primary excess of PTH is associated with increased cardiovascular mortality [24], but in this context P tends to be low. Secondary elevations of PTH in impaired kidney function have been inconsistently associated with mortality. This compensatory mechanism in CKD is triggered when eGFR falls below 47 mL/min [25]. Although PTH levels were not available, the proportion of patients in our cohorts with eGFR below that

threshold was considerably low (4% in RS-I and 2% in RS-II) suggesting that secondary hyperparathyroidism is unlikely to explain our findings. Nevertheless, PTH values seem to rise within normal range in the general population without CKD [26] at higher thresholds of decreasing eGFR (<120 mL/min); whether increasing PTH values within normal range are associated with all-cause mortality in the long term is unclear [27].

Other important players in P homeostasis that might underlie its associations with mortality are the *phosphatonins* FGF23 and α-Klotho [3, 4]. FGF23 is synthesized mainly in osteocytes [5] and requires the presence of α-Klotho to bind to its receptor with high affinity and for signaling [28]. FGF23/α-Klotho axis decreases P through increased urinary phosphate excretion and both molecules are anti-ageing factors [5]. Primary causes of excess FGF23, such as in hereditary hypophosphatemic rickets, have been associated with cardiovascular calcification in cases of excessive phosphate treatment. Secondary FGF23 elevation occurs in CKD at earlier stages than PTH [3, 25] in response to P retention, and it has been linked to increased mortality [29, 30]. Similar to PTH, FGF23 elevations within normal range have been described at high thresholds of eGFR in population without CKD [26]; FGF23 levels have also been associated with mortality in this setting [31]. Nevertheless, FGF23 seems not to induce vascular calcification in most studies [4, 32, 33].

Recently, soluble klotho has been linked to increased mortality in CKD patients [34] although the lack of a validated assay for its measurement might be a concern for some [30].

Another potential confounder could be smoking. Similar to previous reports [7], P was found to be higher in current smokers. Although adjustments for smoking did not alter our analyses, due to heavy current and former smoking in men it is difficult to fully dissect its effects. Nevertheless, in studies combined the stratified analyses by smoking status showed that the associations between P and all-cause and CVD mortality appeared to be of the same direction and similar magnitude

across smoking categories. The group of former smokers - who had similar P as never smokers - displayed the most statistically significant associations possibly due to larger number of subjects in this category. Specifically, P was related to COPD mortality comparably in current and former smokers men from RS-I but only significant in the latter group; a relation in non-smokers could not be tested due to low numbers in this subgroup. Therefore we do not anticipate that current smoking explains the association between P and COPD mortality.

Regarding direct effects, P itself is able to induce vascular calcification, a process with high resemblance to bone ossification and that increases mortality [33, 35]. Several pathways are known such as (a) differential gene expression in vascular smooth muscle cells with up-regulation of markers critical for mineralization [36]; and (b) elastin degradation, thought to be mediated by P induction of matrix metalloproteinase (MMP)-9.

The association we found between P and COPD mortality has never been described in humans before; interestingly there is additional evidence for the pathogenicity of high P stemming from rodent models with *fgf23* or *klotho* knockout. These animals display similar phenotypes characterized by severe hyperphosphatemia and features of premature aging, such as osteoporosis, ectopic calcifications, pulmonary emphysema and short life span [37–39]. Heterozygous klotho mice also display emphysematous lungs. Remarkably, a low phosphate diet is able to alleviate or rescue the phenotype - including the lung emphysema; and a high phosphate diet worsens it [40], strongly suggesting that phosphate itself accelerates ageing [41] and induces alveoli destruction, and that this process can be modified by diet manipulation [40].

A new concept of *phosphotoxicity* as a risk factor for mammalian ageing has emerged lately [3, 40] and there are concerns that increasing phosphate intake through food additives may negatively influence multiple aspects of health [42]. Indeed it has been shown that high absolute P intake was positively related to all-cause mortality - not explained by CVD mortality [11]. Recently, a healthy diet

- according to the Alternate Healthy Eating Index (2010) score - was associated with lower risk of COPD in humans [43]; interestingly in men but not women this beneficial association was driven mostly by a drastic reduction in red and processed meat consumption, expected to contain high phosphate [42]. A positive relation between cured meat intake and COPD risk has previously been reported in cross-sectional (NHANES III) and prospective studies [44, 45]. Importantly, when spirometric definitions for lung volumes and COPD have been applied, cured meat intake has been shown to be negatively associated with lung function, and positively related with COPD risk [44, 46]; the latter study showed that these associations were found predominantly in men. Cured meat consumption has also been shown prospectively to increase the hospital readmission rate in COPD patients [47].

From a mechanistic point of view, previous research [48] has shown that *phosphate is able to directly induce injury in mice and human lung epithelial cells* through increased DNA oxidative stress and apoptosis; indeed phosphate medium is used experimentally to induce oxidative lung injury. Interestingly, α-Klotho exerts protective antioxidant effects against lung injury induced by P [48], hyperoxia, and acute α-Klotho deficiency [49]. These data show that lung tissue is a target for phosphotoxic insult. Remarkably, increased P intake down-regulates α-Klotho expression in rodents [41]; therefore low P diet may be a therapeutic strategy to increase Klotho [3].

A genetic variant associated with low FGF23 was found to be associated with emphysema in smokers with COPD. More studies are needed to elucidate further the underlying mechanisms, especially considering that COPD ranks high in the most common causes of death worldwide.

The reasons for the sex difference between P and mortality are not clear. Interestingly, the vascular calcification induction by P is attenuated by 17β-estradiol, suggesting a potential hormonal reason for this difference [50]. Despite the fact that menopause is characterized by low estradiol levels,

hormone replacement therapy-naïve postmenopausal women with higher 17β-estradiol levels display lower coronary calcification scores than those with lower 17β-estradiol [51]. Additionally, coronary infusion of 17β-estradiol exerts vasodilation in postmenopausal women, but not men [52]. Testosterone and estradiol play important roles as P regulators [19].

Although men had a less healthy profile at baseline than women, multiple adjustments did not abolish our results. Moreover, a previous study showed that P is associated with subclinical atherosclerosis in men (but not women) without prevalent cardiovascular and cerebrovascular disease at baseline [53].

This study has several limitations. 1,25-dihydroxyvitamin D_3 levels were available only in a subgroup. PTH and FGF23 measurements were not available and it is known that kidney function in elderly can be misclassified even by eGFR. Our findings cannot be generalized to other ethnicities other than European Caucasians. Nevertheless, there are several strengths, such as the availability of two well-characterized cohorts with long follow-up, the detailed information on cause-specific mortality and the availability of multiple potential confounders. The completeness of follow-up was high (94 and 92% in RS-I and RS-II) indicating that obtained estimates are valid.

In conclusion, we found that higher P is associated with increased all-cause mortality and cause-specific mortality due to CVD, COPD and other causes in elderly men but not in women, adding more evidence for a modification of these associations by sex. We hereby provide evidence to support that the concept of phosphotoxicity also among non-CKD general population deserves further attention and, if causally related, it occurs independently of vitamin D levels and kidney function. Our study suggests that moderation of phosphate intake might be relevant also in non-CKD population for healthy ageing. Finally, we consider that the available evidence calls for a review of the currently accepted normal range of P. Further research is needed to clarify the underlying mechanisms, especially for COPD mortality, and to elucidate the reasons for the sex difference in the

association of P with mortality.

Acknowledgments The authors thank the participants and staff of the research center of the Rotterdam Study. The authors also acknowledge Dr. F. Rivadeneira, for his critical review of the manuscript. 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 (Population Screening Act WBO, License Number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/net work/primary/en/) 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.

Author contribution Dr. Zillikens and N. Campos-Obando are the study guarantors and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: N. Campos-Obando and Dr. Zillikens. Acquisition of data: Prof. Hofman, Dr. Zillikens, Prof. Uitterlinden, Prof. Stricker, Prof. Brusselle, Prof. Franco, Dr. Lahousse. Analysis and interpretation of data: N. Campos-Obando and Dr. Zillikens. Drafting of the manuscript: N. Campos-Obando and Dr. Zillikens. Critical review of the manuscript for important intellectual content: all authors. Statistical analyses: N. Campos-Obando. Obtained funding: Prof. Hofman, Prof. Uitterlinden. Administrative, technical and material support: Dr. Zillikens, Prof. Uitterlinden. Study supervision: Dr. Zillikens.

Funding The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The funding sources had no influence in the study design, collection, analysis, interpretation of data, writing of the report and in the decision to submit the article.

References

1. **Koeppen BM, Stanton BA**. The renal system. In: Koeppen BM, Stanton BA editors. *Berne & Levy physiology*, 6th edn. Philadelphia, PA; 2010. p. 557-636.

2. **Shimada T, Kakitani M, Yamazaki Y, et al**. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004;113(4):561–8.

3. **Hu MC, Shiizaki K, Kuro-o M, Moe OW**. Fibroblast growth factor 23 and Klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* 2013;75:503–33.

4. **Lim K, Lu TS, Molostvov G, et al**. Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* 2012;125(18):2243–55.

5. **Martin A, David V, Quarles LD**. Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* 2012;92(1):131–55.

6. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK**. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998;31(4):607–17.

7. **Dhingra R, Sullivan LM, Fox CS, et al**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007;167(9):879–85.

8. **Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G, Cholesterol And Recurrent Events Trial Investigators**. Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005;112(17):2627–33.

9. **Onufrak SJ, Bellasi A, Cardarelli F, et al**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009;169(1):67–77.

10. **Chang AR, Grams ME**. Serum phosphorus and mortality in the Third National Health and Nutrition Examination Survey (NHANES III): effect modification by fasting. *Am J Kidney Dis* 2014;64(4):567–73.

11. **Chang AR, Lazo M, Appel LJ, Gutie´rrez OM, Grams ME**. High die-

tary phosphorus intake is associated with all-cause mortality: results from NHANES III. *Am J Clin Nutr* 2014;99(2):320–7.

12. **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.

13. **Bolland MJ, Grey AB, Ames RW, et al**. The effects of seasonal variation of 25-hydroxyvitamin D and fat mass on a diagnosis of vitamin D sufficiency. *Am J Clin Nutr* 2007;86(4):959–64.

14. **Tomson J, Emberson J, Hill M, et al**. Vitamin D and risk of death from vascular and non-vascular causes in the Whitehall Study and meta-analyses of 12000 deaths. *Eur Heart J* 2013;34(18):1365–74.

15. **Terzikhan N, Verhamme KM, Hofman A, Stricker BH, Brusselle GG, Lahousse L**. Prevalence and incidence of COPD in smokers and non-smokers: the Rotterdam Study. *Eur J Epidemiol* 2016;31(8):785–92.

16. **Stevens PE, Levin A, Kidney Disease: Improving Global Outcomes Chronic Kidney Disease Guideline Development Work Group Members**. Evaluation and management of chronic kidney disease: synopsis of the kidney disease: improving global outcomes 2012 clinical practice guideline. *Ann Intern Med* 2013;158(11):825–30.

17. **Levey AS, Stevens LA, Schmid CH, CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration), et al**. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150(9):604–12.

18. **Campos-Obando N, Castano-Betancourt MC, Oei L, et al**. Bone mineral density and chronic lung disease mortality: the Rotterdam Study. *J Clin Endocrinol Metab* 2014;99(5):1834–42.

19. **Meng J, Ohlsson C, Laughlin GA, Osteoporotic Fractures in Men (MrOs) Study Group, et al**. Associations of estradiol and testosterone with serum phosphorus in older men: the Osteoporotic Fractures in Men study. *Kidney Int* 2010;78(4):415–22.

20. **Collet D**. Modelling survival data in medical research. 3rd ed. *London: Chapman & Hall/CRC*; 2014.

21. **Putter H, Fiocco M, Geskus RB**. Tutorial in biostatistics: competing

risks and multi-state models. *Stat Med* 2007;26(11):2389–430.

22. **Austin PC, Fine JP**. Accounting for competing risks in randomized co trolled trials: a review and recommendations for improvement. *Stat Med* 2017;36(8):1203–9.

23. ***Lee DM, Vanderschueren D, Boonen S, European Male Ageing Study Group, et al***. Association of 25-hydroxyvitamin D, 1,25- dihydroxyvitamin D and parathyroid hormone with mortality among middle-aged and older European men. *Age Ageing* 2014;43(4):528–35.

24. **Nilsson IL, Yin L, Lundgren E, Rastad J, Ekbom A**. Clinical presentation of primary hyperparathyroidism in Europe–nationwide cohort analysis on mortality from nonmalignant causes. *J Bone Miner Res* 2002;17(Suppl 2):N68–74.

25. **Isakova T, Wahl P, Vargas GS, et al**. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011;79(12):1370–8.

26. **Dhayat NA, Ackermann D, Pruijm M, et al**. Fibroblast growth factor 23 and markers of mineral metabolism in individuals with preserved renal function. *Kidney Int* 2016;90(3):648–57.

27. **Yang B, Lu C, Wu Q, Zhan J, Zhao H, Cao Y**. Parathyroid hormone, cardiovascular and all-cause mortality: a meta-analysis. *Clin Chim Acta* 2016;455:154–60.

28. **Urakawa I, Yamazaki Y, Shimada T, et al**. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006;444(7120):770–4.

29. **Yang H, Luo H, Tang X, et al**. Prognostic value of FGF23 among patients with end-stage renal disease: a systematic review and meta-analysis. *Biomark Med* 2016;10(5):547–56.

30. **Munoz Mendoza J, Isakova T, Cai X, CRIC Study Investigators, et al**. Inflammation and elevated levels of fibroblast growth factor 23 are independent risk factors for death in chronic kidney disease. *Kidney Int*. 2017;91(3):711–9.

31. **Qin Z, Liu X, Song M, et al**. Fibroblast growth factor 23 as a predictor of cardiovascular and all-cause mortality in prospective studies. *Atherosclerosis* 2017;261:1–11.

32. **Scialla JJ, Lau WL, Reilly MP, Chronic Renal Insufficiency Cohort Study Investigators, et al**. Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. *Kidney Int* 2013;83:1159–68.

33. **Yamada S, Giacelli CM**. Vascular calcification in CKD-MBD: roles for phosphate, FGF23 and Klotho. *Bone* 2017;100:87–93.

34. **Marc¸ais C, Maucort-Boulch D, Drai J, ARNOGENE project, et al**. Circulating klotho associates with cardiovascular morbidity and mortality during hemodialysis. *J Clin Endocrinol Metab* 2017;102(9):3154–61.

35. **Wu M, Rementer C, Giachelli CM**. Vascular calcification: an update on mechanisms and challenges in treatment. *Calcif Tissue Int* 2013;93(4):365–73.

36. **Roma´n-Garcı´a P, Carrillo-Lo´pez N, Ferna´ndez-Martı´n JL, Naves-Dı´az M, Ruiz-Torres MP, Cannata-Andı´a JB**. High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. *Bone* 2010;46(1):121–8.

37. **Kuro-o M, Matsumura Y, Aizawa H, et al**. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997;390(6655):45–51.

38. **Nakatani T, Sarraj B, Ohnishi M, et al**. In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23)-mediated regulation of systemic phosphate homeostasis. *FASEB J* 2009;23(2):433–41.

39. **Suga T, Kurabayashi M, Sando Y, et al**. Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* 2000;22(1):26–33.

40. **Ohnishi M, Razzaque MS**. Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. *FASEB J* 2010;24(9):3562–71.

41. **Morishita K, Shirai A, Kubota M, et al**. The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. *J Nutr* 2001;131(12):3182–8.

42. **European Food Safety Authority**. Assessment of one published review on health risks associated with phosphate additives in food. *EFSA Journal* 2013;11:3444. https://doi.org/10.2903/j.efsa.2013.3444.

43. **Varraso R, Chiuve SE, Fung TT, et al**. Alternate Healthy Eating Index

2010 and risk of chronic obstructive pulmonary disease among US women and men: prospective study. *BMJ* 2015;350:h286.

44. **Jiang R, Paik DC, Hankinson JL, Barr RG**. Cured meat consumption, lung function and chronic obstructive pulmonary disease among United States adults. *Am J Respir Crit Care Med* 2007;175(8):798–804.

45. **Varraso R, Jiang R, Barr RG, Willet WC, Camargo CA Jr**. Prospective study of cured meats consumption and risk of chronic obstructive pulmonary disease in men. *Am J Epidemiol* 2007;166(12):1438–45.

46. **Okubo H, Shaheen SO, Ntani G, Hertfordshire Cohort Study Group, et al**. Processed meat consumption and lung function: modification by antioxidants and smoking. *Eur Respir J* 2014;43(4):972–82.

47. **de Battle J, Mendez M, Romieu I, PAC-COPD Study Group, et al**. Cured meat consumption increases risk of readmission in COPD patients. *Eur Respir J* 2012;40(3):555–60.

48. **Ravikumar P, Ye J, Zhang J, et al**. a-Klotho protects against oxidative damage in pulmonary epithelia. *Am J Physiol Lung Cell Mol Physiol* 2014;307(7):L566–75.

49. **Ravikumar P, Li L, Ye J, et al**. aKlotho deficiency in acute kidney injury contributes to lung damage. *J Appl Physiol* 2016;120(7):723–32.

50. **Rzewuska-Lech E, Jayachandran M, Fitzpatrick LA, Miller VM**. Differential effects of 17beta-estradiol and raloxifene on VSMC phenotype and expression of osteoblast-associated proteins. *Am J Physiol Endocrinol Metab* 2005;289(1):E105–12.

51. **Jeon GH, Kim SH, Yun SC, Chae HD, Kim CH, Kang BM**. Association between serum estradiol levels and coronary artery calcification in postmenopausal women. *Menopause* 2010;17(5):902–7.

52. **Collins P, Rosano GM, Sarrel PM, et al**. 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. *Circulation* 1995;92(1):24–30.

53. **Onufrak SJ, Bellasi A, Shaw LJ, et al**. Phosphorus levels are associated with subclinical atherosclerosis in the general population. *Atherosclerosis* 2008;199(2):424–31.

Supplementary Material

Supplementary Table 1. Goodness of fit among parametric and semiparametric models for the association between serum phosphate levels and mortality in men from RS-I, according to AIC and BIC criteria:

	AIC	BIC
Parametric models		
Weibull	**3060.27**	**3097.81**
Exponential	3246.42	3278.59
Gompertz	3065.36	3102.9
Lognormal	3194.43	3231.97
Loglogistic	3106.07	3143.62
Generalized gamma	3060.31	3103.22
Semiparametric model		
Cox	10865.15	10891.96

AIC: Akaike information criteria; BIC: Bayesian information criteria

Supplementary Table 2. Competing-risk results for serum phosphate levels and cause-specific mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012:

		Individual cohorts			Studies combined	
	Cohort	**n**	**SHR[*] (95% CI)**	*p*	**SHR*,† (95% CI)**	*p*
CVD	RS-I	266	**1.60** (1.14-2.24)	0.006	**1.50** (1.12-2.02)	0.006
	RS-II	77	1.24 (0.68-2.26)	0.488		
Cancer	RS-I	243	1.20 (0.92-1.56)	0.179	1.10 (0.87-1.39)	0.409
	RS-II	98	0.83 (0.51-1.34)	0.438		
External	RS-I	18	1.18 (0.45-3.13)	0.736	0.73 (0.33-1.59)	0.426
	RS-II	09	0.30 (0.08-1.11)	0.071		
Infectious	RS-I	56	0.81 (0.43-1.50)	0.497	0.79 (0.44-1.41)	0.427
	RS-II	09	0.66 (0.13-3.48)	0.628		
Dementia	RS-I	52	1.31 (0.83-2.06)	0.240	1.30 (0.84-2.01)	0.235
	RS-II	13	1.21 (0.28-5.20)	0.797		
Lung	RS-I	42	1.49 (0.83-2.70)	0.184	1.48 (0.87-2.50)	0.149
	RS-II	15	1.42 (0.43-4.64)	0.564		
Other	RS-I	133	1.27 (0.88-1.84)	0.202	**1.40** (1.01-1.93)	0.043
	RS-II	40	1.91 (0.98-3.74)	0.059		

[*] Subhazard ratios from competing risks models, interpreted as the relative increase in the incidence of the event of interest per 1 mg/dL (0.32 mmol/L) increase in serum phosphate in the presence of competing risks.

[†] Studies combined from meta-analyses using fixed-effect models

Supplementary Table 3. Competing-risks results for serum phosphate levels and COPD mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012:

		Individual cohorts		Studies combined	
	n	**SHR[*] (95% CI)**	***p***	**SHR[*,†] (95% CI)**	***p***
RS-I	28	**2.42** (1.61-3.65)	<0.001	**2.42** (1.62-3.63)	<0.001
RS-II	05	2.54 (0.20-32.8)	0.475		

[*] Subhazard ratios from competing risks models, interpreted as the relative increase in the incidence of the event of interest per 1 mg/dL (0.32 mmol/L) increase in serum phosphate in the presence of competing risks.

[†] Studies combined from meta-analyses using fixed-effect models

Supplementary Table 4. Serum phosphate levels within normal range and cause- specific mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012:

			Individual cohorts		Studies combined	
	Cohort	**n**	**HR[*] (95% CI)**	***p***	**HR[†] (95% CI)**	***p***
CVD	RS-I	249	**1.72** (1.22-2.42)	0.002	**1.60** (1.19-2.16)	0.002
	RS-II	73	1.27 (0.68-2.37)	0.449		
Cancer	RS-I	230	1.41 (0.99-2.01)	0.056	1.33 (0.98-1.80)	0.066
	RS-II	82	1.13 (0.63-2.01)	0.681		
External	RS-I	18	1.24 (0.33-4.59)	0.750	0.80 (0.25-2.58)	0.703
	RS-II	8	0.14 (0.01-1.84)	0.134		
Infectious	RS-I	54	0.61 (0.28-1.34)	0.220	0.58 (0.28-1.20)	0.142
	RS-II	9	0.37 (0.04-.3.04)	0.358		
Dementia	RS-I	51	1.61 (0.75-3.47)	0.222	1.63 (0.81-3.27)	0.171
	RS-II	12	1.72 (0.32-9.33)	0.530		
Lung	RS-I	39	**2.88** (1.21-6.89)	0.017	**2.31** (1.11-4.83)	0.026
	RS-II	15	1.32 (0.33-5.32)	0.691		
Other	RS-I	127	1.53 (0.94-2.50)	0.085	**1.66** (1.09-2.53)	0.018
	RS-II	38	2.11 (0.93-4.82)	0.075		

[*] Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels constrained to normal values (2.5-4.5 mg/dL; 0.81-1.45 mmol/L)

[†] Studies combined from meta-analyses using fixed-effect models

Supplementary Table 5. Serum phosphate levels within normal range and chronic obstructive pulmonary disease (COPD) mortality in men from RS-I and RS-II, adjusted for age, BMI and smoking, follow-up until year 2012:

	Individual cohorts			Studies combined	
	n	HR* (95% CI)	*p*	HR† (95% CI)	*p*
RS-I	27	**7.18** (2.53-20.4)	<0.001	**6.22** (2.39-16.2)	<0.001
RS-II	05	2.98 (0.28-31.9)	0.367		

* Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels constrained to normal values (2.5-4.5 mg/dL; 0.81-1.45 mmol/L)

† Studies combined from meta-analyses using fixed-effect models

Supplementary Table 6. Serum phosphate levels and all-cause and CVD mortality in men from RS-I and RS-II stratified by smoking status, adjusted for age and BMI, follow-up until year 2012:

	All-cause mortality			CVD mortality		
	n	HR*(95% CI)	*p*	n	HR*(95% CI)	*p*
RS-I						
Never smoker	57	**2.11** (1.04-4.26)	0.038	16	1.17 (0.28-4.88)	0.827
Former smoker	543	**1.66** (1.36-2.04)	<0.001	184	**1.80** (1.28-2.52)	0.001
Current smoker	210	1.36 (0.99-1.86)	0.059	66	**1.97** (1.11-3.51)	0.021
RS-II						
Never smoker	29	0.95 (0.37-2.47)	0.923	08	1.45 (0.25-8.25)	0.674
Former smoker	162	1.11 (0.76-1.63)	0.570	47	1.65 (0.81-3.33)	0.165
Current smoker	71	1.31 (0.76-2.26)	0.331	22	0.74 (0.27-2.05)	0.563
Studies combined†						
Never smoker	86	1.59 (0.90-2.80)	0.109	24	1.27 (0.42-3.85)	0.667
Former smoker	705	**1.52** (1.27-1.82)	<0.001	231	**1.77** (1.30-2.40)	<0.001
Current smoker	281	**1.35** (1.02-1.77)	0.032	88	1.55 (0.94-2.56)	0.085

* Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels

† Studies combined from meta-analyses using fixed-effect models

Supplementary Table 7. Serum phosphate levels and COPD mortality in men from RS-I stratified by smoking status, adjusted for age and BMI, follow-up until year 2012:

		COPD mortality	
	n	**HR* (95% CI)**	***p***
RS-I			
Never smoker	01	n/a	
Former smoker	20	**5.59** (2.21-14.1)	<0.001
Current smoker	07	2.64 (0.38-18.3)	0.326

* Hazard ratios from Weibull models, expressed per 1 mg/dL (0.32 mmol/L) increase in phosphate levels

6

Genetic evidence for a causal role of serum phosphate in coronary artery calcification: The Rotterdam Study

Genetic evidence for a causal role of serum phosphate in coronary artery calcification: The Rotterdam Study

Authors:

Campos-Obando N, Kavousi M, Medina-Gómez MC, van der Eerden BC, Bos D, Franco OH, Uitterlinden AG, Zillikens MC

Status:

Manuscript ready for submission.

Abstract

Background: Coronary artery calcification (CAC) is a vascular calcification process specific of atherosclerosis, located mainly in the intima and predictive of coronary heart disease. Hyperphosphatemia (hyperP) has been associated with CAC mostly in kidney disease, but the association in normal phosphate (P) setting is unclear. Moreover, it is unknown if this outcome displays sex differences, common in P-related associations. Our objectives were to evaluate the association between P and CAC and potential sex-differences in a population-based setting, assessing causality through Mendelian Randomization (MR).

Methods: serum P and electron-beam computed tomography- CAC were assessed in Rotterdam Study I. Phenotypic associations were tested through linear models adjusted for age, body mass index, blood pressure, smoking, prevalent cardiovascular disease (CVD) and diabetes mellitus, 25-hydroxyvitamin D, total calcium, C-reactive protein, glucose and cholesterol:HDL ratio. MR was implemented through an allele score including eight P single nucleotide polymorphisms (SNPs) reported by the European CHARGE Consortium and BioBank Japan Project. MR assumptions were assessed through SNP strength (relevance); regression with potential confounders (independence) and testing or allowance of pleiotropic effects (exclusion restriction). Models included two-stage least square, adaptive lasso and variational Bayes.

Results: In phenotypic analyses, P was related to CAC with evidence of sex interaction ($p_{interaction}$=0.005) [men β:0.38 (95% CI: 0.26-0.51), p=5x10^{-9}, n=878; women β:0.20 (0.06-0.34), p=0.005, n=1011]; exclusion of hyperP, chronic kidney disease (CKD: eGFR<60 mL/min) and prevalent CVD yielded [men β:0.46 (0.29-0.63), p=1x10^{-7} ,n=592; women β:0.19 (0.01-0.36), p=0.033, n=766]. In MR analyses, *instrumented* P through unweighted allele score was related to CAC (sex-combined β:0.82 (0.04-1.59), p=0.039, n=1693) – even after exclusion of hyperP, CKD and prevalent CVD (sex-combined β:1.55 (0.45-2.65), p=0.006, n=1220). No evidence of invalid instruments was found. Allowance of pleiotropy for almost half the SNPs still yielded significant results [sex-combined posterior mean: 0.82 (95% Credible Interval: 0.47-1.26)].

Conclusions: P is phenotypically related to CAC in the general population with a stronger effect in men. MR findings support a causal relation; also for P and CAC in subjects *without* hyperP, CKD and CVD. Further research into underlying mechanisms and sex differences is needed. Our findings might have an impact in public health.

Introduction

Arterial calcification is defined as the deposition of calcium and phosphate in the wall of arteries (1). It was considered a passive consequence of aging until identified as a risk factor for cardiovascular (CV) events (2). Current evidence supports that a complex cellular-mediated process underlies it (3). At least, two different layers can calcify: the intima, characteristic of atherosclerosis; and the media, typical of chronic kidney disease (CKD). The arterial mineralization process can reach hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], as is found in bone.

Coronary artery calcification (CAC) is one of the most studied calcification processes because of its specificity for atherosclerosis, its correlation to plaque burden (4) and its ability to predict CV events (2, 5). In 1912 Faber reported that calcification in the coronary bed *did not occur in the medial layer* (2); currently it is widely accepted that CAC occurs mainly in the intima (6). Fragmented calcification is frequently encountered in culprit plaques (4).

Calcification mechanisms are multifactorial but ectopic bone formation is currently considered the basis of CAC, as several cells are able to *transdifferentiate* into osteoblast-like cells and mineralize, such as calcifying vascular cells and pericytes (7), consistent with findings of trabeculae, Haversian canals (7) and a calcium: phosphate ratio of 1.66:1.

Serum phosphate (P) was related to arterial calcification in several human and animal disorders (8, 9), where genetically-induced severe hyperphosphatemia (hyperP) leads to extensive calcification. HyperP-induced calcification was described as the main mechanism of increased mortality in CKD (10). Similarly, the role of P in CAC has been restricted mainly to hyperP (11). Nevertheless, it has been reported that *increasing yet normal* P is also a risk factor for CV morbidity and mortality in the general population (12, 13). These findings have been consistent although a clear sex difference with stronger associations in men became evident (13, 14). The underlying mechanisms remain unexplained.

Two non-mutually exclusive mechanisms have been described for P in CAC: a) passive deposition of calcium phosphate - inhibited by pyrophosphate (PPi); b) active induction of osteoblastic differentiation of vascular cells (15). Additionally, P is regulated by several factors that exert inductive (parathyroid hormone - PTH - and 1,25-hydroxyvitamin D) or protective effects (α-klotho and FGF23) on arterial calcification (16, 17).

As literature on P and CAC in the general population is scarce (18, 19), we aimed to analyze this association in the population-based Rotterdam Study and test for potential sex differences. Because results from epidemiologic associations can be affected by reverse causation and unmeasured confounding, we also aimed to test for causality applying Mendelian Randomization, an Econometrics technique whereby genetic variants are used to instrument the exposure with the purpose of avoiding these sources of bias. If rigorously applied, significant MR results can be interpreted as evidence of causality of the exposure in the outcome (20).

Materials and methods

Study population

The Rotterdam Study (RS) is a prospective cohort study designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described elsewhere (21). The Rotterdam Study I cohort (RS-I) initiated in 1990 and consisted of 7983 participants. All subjects were >55 years at recruitment and reside in Ommoord, a district in Rotterdam and have been assessed at baseline and through follow-up visits. During the second follow-up visit, fasting P and CAC were assessed. A total of 1889 subjects with both measurements available were included in the analyses. 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 (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register

and into the WHO International Clinical Trials Registry Platform under shared catalogue number NTR6831. All participants provided written informed consent to participate and to have their information obtained from physicians.

Coronary calcification assessment

Coronary artery calcification was visualized using electron-beam Computed Tomography (EBT; C-150 Imatron Scanner, GE Healthcase, South San Francisco, CA). From the level of the aortic root through the heart, 38 images were obtained with a 100-ms scan time and a 3-mm slice thickness. During one breath hold, images were acquired at 80% of the cardiac cycle by using echocardiographic triggering. Quantification of coronary calcification was performed with AccuImage software (AccuImage Diagnostics Corporation, South San Francisco, CA). The presence of calcification was defined as a *minimum* of 2 adjacent pixels (area=0.65 mm^2) with a density > 130 HU. Following Agatston's method, calcium scores were calculated by multiplying the area in mm^2 of individual calcified lesions with a factor based on the peak density of the lesion. The total score for the entire epicardial coronary vascular system comprised the sum of the scores for all individual lesions. Scores were log transformed (+1) to achieve normality.

Laboratory measurements

Fasting blood samples were obtained in the second follow-up visit and serum P was measured with a method based on the formation of ammonium phosphomolybdate, that corresponds to the inorganic fraction of total phosphorus. Total calcium was assessed through a colorimetric o-cresolphthalein complex one method (Merck Diagnostica, Amsterdam, The Netherlands). Levels of 25-hydroxyvitamin D (25OHD) were determined through an electrochemiluminescense immunoassay, adjusting for seasonality through cosinor models. Creatinine was determined through a sarcosine-based colorimetric assay and standardized; subsequently the Chronic Kidney Disease Epidemiology Collaboration equations were applied to estimate glomerular filtration rate (eGFR). C-reactive protein (CRP), glucose,

cholesterol and alkaline phosphatase (ALP) levels were measured through standard methods (22, 23).

Ionized calcium was measured through a colorimetric detection assay using Hitachi 917 (Roche, Mannheim, Germany). Assessments for ALP and ionized calcium were done at baseline visit and therefore are not simultaneous to P.

Genotyping

Participants were genotyped in the Illumina HumanHap550 BeadChip SNP array. Variants were filtered on call rate <98%, MAF<0.01 and HWE $P<10^{-6}$, and imputed to the Haplotype Reference consortium panel, release 1.1. We selected as P genetic instruments single nucleotide polymorphisms (SNPs) reported by the GWAS (Genome Wide Association Study) catalogue. Variants selected for analyses were checked for Hardy Weinberg equilibrium (HWE p>0.05) for genotyped SNPs, imputation quality for imputed SNPs (R^2 >0.8) and allele frequencies for palindromic SNPs to decrease the possibility of strand inconsistencies.

Other covariates

Prevalent cardiovascular disease has been defined as prevalent myocardial infarction, revascularization, stroke and heart failure (24). Prevalent diabetes mellitus, blood pressure, smoking and body mass index assessments have been previously described (25).

Statistical analysis

Phenotypic associations

The association between P and CAC was assessed through generalized linear models. The analysis was stratified according to eGFR (< 60 mL/min: chronic kidney disease (CKD)). P was assessed continuously and in quintiles. We tested

if the calcium*phosphate product (Ca*P) was associated to CAC, as previously reported in CKD (26). We assessed the association between P and binary CAC through prevalence ratios, applying a classification relevant for clinical practice (27). When a sex-difference in P and CAC was confirmed we performed sex-stratified analysis. Because calcium is synergistic to P in arterial calcification (26), we assessed the relation between P and ionized calcium.

Model I included adjustments for age, BMI and smoking. Model II included also blood pressure, 25OHD, total serum calcium, CRP, total cholesterol:HDL ratio and glucose levels, prevalent CVD and diabetes mellitus.

Sensitivity analyses

We restricted the analyses to subjects without hyperphosphatemia (hyperP: P>1.45 mmol/L=4.5 mg/dL), without CKD and without prevalent CVD. To explore for interaction, we created 25OHD categories splitting at 48 nmol/L - a threshold related to CVD in Dutch (28). We assessed whether serum tissue non-specific alkaline phosphatase (ALP) was related to CAC, as ALP generates P through hydrolysis of inorganic pyrophosphate (29) - a potent arterial calcification inhibitor - and has been independently related to CAC in the general population, in CVD and in CKD (29).

Mendelian randomization

To test for causality (20) we assessed whether P - instrumented through genetic variants (SNPs) - was associated with CAC. We selected 8 SNPs from the GWAS catalogue, assumed an additive model and built a P-increasing allele score (30) aligning the alleles: a higher score predicted a higher P. Scores (as a single instrument) are associated with lower risk of weak instrument bias than the simultaneous use of multiple SNPs (30).

The SNPs included in the score derived from:

a) A GWAS meta-analysis (31) within CHARGE Consortium - composed of individuals of European ancestry;
b) A recent GWAS (32) within the Biobank Japan Project (BBJ) - composed of individuals of East Asian ancestry.

The rationale for including trans-ethnic SNPs in the score laid in a) the shared allelic spectrum correlation between East Asians and Europeans (~r = 0.687) (32); b) the hypothesis that common variants (MAF>5%) segregated tens of thousands of years ago and are therefore expected to be similarly distributed across populations (33) and c) scores identified in one ancestry might be useful in different populations (34).

To properly account for uncertainty in imputed SNPs, genotypes were extracted from dosage files and therefore its values span between 0-2, reflecting the probability of getting up to 2 risk (P-increasing) alleles. If the SNP was genotyped we report HWE test; otherwise the imputation quality (R^2) is displayed.

Palindromic SNPs were checked for allele frequency concordance between RS and the GWAS catalogue. In addition, one SNP mapping to the non pseudoautosomal region of the X-chromosome was coded as 0/2 in men (for 0/1 risk allele) and as 0/1/2 in women, following recent guidelines when assuming a pattern of X-chromosome inactivation (35).

Inclusion of correlated SNPs - in linkage disequilibrium - is a potential source of bias in standard MR analyses without covariance matrix, leading to increased type I error rates. Therefore, we included only independent SNPs in the score - setting a threshold of $r^2<0.01$.

We *instrumented* P levels through the score and tested for causality applying a two stage least square regression - *tsls*, where first stage regresses the exposure

on the instruments and second stage regresses the outcome on the fitted values of the exposure estimated in first stage (30). We assessed MR assumptions (36) as follows:

1. Assumption N°1 (the instrument [=SNP/score] must be associated with the exposure, *relevance* condition): we regressed P levels on P SNPs/score in the population with P levels available and with P and CAC levels available. We considered *β*, *p* and F-statistic: a higher F-statistic reflects a better instrument but no cut-offs should be made, as bias is a continuous phenomenon (20).

2. Assumption N°2 (the instrument must not be associated with potential confounders, *independence* condition): we regressed potential confounders on P scores. Naturally, it is not possible to assess the association of instruments with unmeasured confounders.

3. Assumption N°3 (the instrument must be related to the outcome only through the exposure, meaning the absence of horizontal pleiotropy, *exclusion-restriction* condition): to test this assumption, both a frequentist and a Bayesian approach were applied, the latter as sensitivity analysis. MR-Egger regression was not applied due to our one-sample setting and its low statistical power (37) when the SNP-exposure associations are homogenous. We implemented instead an adaptive lasso regression (sivreg, Stata) that provides estimates while allowing less than 50% of instruments to be invalid by horizontal pleiotropy (38). The modification of lasso is relevant as variable detection of invalid instruments might not be appropriate when some SNPs are strongly associated with the exposure (high F-statistic), as is the case.

Analyses were performed with both unweighted and weighted scores; *internal weights derived from the data were avoided* due to the severe bias that this approach induces (30); weights were derived instead from CHARGE GWAS and BioBank Japan Project. However, as the Rotterdam Study represented ~40% of the CHARGE GWAS (31), we based the statistical inference from the unweighted

score.

To test if results from the score were driven by only one SNP, we applied the *leave-one-out approach*, excluding one SNP at a time from the score and testing for each reduced score if instrumented P was still related to CAC. This *penalization* technique is considered a robust method (39): if results are driven by only one SNP a high index of pleiotropy should be suspected and properly assessed.

Similar to phenotypic associations, we stratified analyses excluding participants with hyperphosphatemia, CKD and prevalent CVD.

Sensitivity analysis

In addition of applying a single score, we instrumented P through the combination of all SNPs simultaneously (joint instruments analyses). This approach might have more power but might suffer from weak instrument bias (30). We applied in this setting the Sargan test - an overidentification test - to assess whether all intruments included in the regression are valid in linear combination (40) and not correlated with error terms. Additionally, Sargans' provides a test of heterogeneity among instruments (39).

Consistent with ancestry of participants, we built a score derived only from SNPs from CHARGE metaanalysis (EUR-score), and tested for reproducibility of results.

Finally, we applied a Bayesian approach designed for one-sample setting that allows several SNPs to exert pleiotropic effects and that does not rely in the assumption of no correlation between SNP strength and pleiotropic effects. The method allows the implementation of pleiotropic effects for a subset (49%) of SNPs - incorporated in their prior distribution - and applies variational Bayes through a modified Markov Chain Monte Carlo to estimate the posterior mean and its 95% Credible Interval (41). Genotypes were centered to improve convergence.

Analyses were performed after imputing missing values through multiple imputation with chained equations. We used SPSS (version 21.0, Armonk, NY: IBM Corp), Stata (version 15, College Station TX: Stata Corp LP) and R (version 3.5.0; Vienna, Austria). A two-sided $p<0.05$ was considered significant.

Results

The general characteristics of the study population are shown in **Table 1**. P mean levels were higher in women: P_{women}: 1.17 mmol/L (3.63 mg/dL); P_{men}: 1.01 mmol/L (3.14 mg/dL); $p_{t\text{-}test}< 0.001$ (data not shown). CAC score median levels were higher in men (CAC_{men}: 310.7; CAC_{women}: 54.1; $p_{M\ Whitney} <0.001$). Total calcium and calcium*phosphate product was positively related to P in both genders while ionized calcium was negatively related to P in women only.

Results are expressed per 1 SD increase of P in mmol/L (0.14 mmol/l=0.43 mg/dL). Results expressed in conventional units (1 mg/dL) are shown in the Online Data Supplement.

Covariates missingness was less than 5%.

Results from phenotypic associations

CAC as continuous trait

Table 2a shows that P was related to CAC in sex-combined analyses after adjustments for Model I (β: 0.31 (0.21-0.41), $p<<0.001$ [$p=6x10^{-10}$], n=1889). We performed sex-stratified analyses due to a significant sex interaction ($p_{interaction}=0.005$) driven by a stronger association in men (β:0.45 (0.32-0.58), $p<<0.001$ [$p=2.9x10^{-11}$], n=878) than women (β:0.18 (0.04-0.32), $p=0.010$; n=1011). Further adjustments (Model II) induced a slight attenuation in men (men β:0.38 (0.26-0.51), [$p=5x10^{-9}$]; women β:0.20 (0.06-0.34), $p=0.005$).

The stratified analyses (**Table 2b**) showed that P was associated with CAC across the spectrum of kidney function in men [eGFR≥ 60 mL/min β:0.43 (0.28-0.59), p<0.001, n=768; eGFR<60mL/min β:0.52 (0.29-0.74), p<0.001, n=110]; while in women this association was constrained to normal eGFR [eGFR≥ 60 mL/min β:0.18 (0.03-0.33), p=0.020, n=877; eGFR<60mL/min β:0.17 (-0.23-0.58), p=0.397, n=134]. Adjustments for Model II induced a slight attenuation in men [eGFR ≥ 60 mL/min β:0.38 (0.23-0.52), p<0.001; eGFR<60 mL/min β:0.39 (0.16-0.63), p=0.001].

The analyses in quintiles suggested a threshold for the association of P and CAC (**Table 3**): setting the first quintile as reference, men with P above 1.09 mmol/L (3.35 mg/dL) displayed a significant trend for higher CAC (β for fourth quintile: 0.80 (0.39-1.21), p<0.001; β for fifth quintile:1.09 (0.68-1.50), p<0.001; p_{trend} <<0.001 [1x10^{-8}], n=878). For women, the threshold was above 1.37 mmol/L (4.24 mg/dL) (β for fifth quintile 0.61 (0.17-1.05), p=0.007; p_{trend} =0.004, n=1011).

Similarly, the results of the product with CAC (**Table 4**) suggested threshold values: setting the first quintile as reference, men with product above 2.44 mmol^2/L^2 (30.2 mg^2/dL^2) displayed a significant trend for higher CAC (β for third quintile: 0.48 (0.08-0.89), p=0.020, β for fourth quintile: 0.76 (0.35-1.16), p<0.001, β for fifth quintile: 1.15 (0.74-1.56), p<0.001; p_{trend} <<0.001 [2x10^{-10}], n=878). In women, the threshold was above 3.05 mmol^2/L^2 (37.7 mg^2/dL^2) (β for fourth quintile: 0.47 (0.03-0.90), p=0.035, β for fifth quintile 0.60 (0.16-1.04), p=0.008; p_{trend} =0.001, n=1011).

Alkaline phosphatase was not related to CAC (men β: -0.001 (-0.009 to 0.006), p=0.731, n=612; women β: -0.003 (-0.006 to 0.001), p=0.163, n=800) (data not shown).

CAC as binary trait

After adjustments for Model I (**Supplementary Table 1**), each SD increase in P

(0.14 mmol/L = 0.43 mg/dL) was related to an increased prevalence ratio (PR) for CAC>100 of 9% in men ((6%-12%), $p<<0.001[1x10^{-7}]$) and 7% in women ((1%-15%), $p=0.043$); a PR for CAC>300 of 11% in men only ((7%-16%), $p<<0.001[2x10^{-7}]$); a PR for CAC>400 of 13% in men only ((8%-18%), $p<<0.001[1x10^{-8}]$), and a PR for CAC>1000 of 19% in men ((14%-25%), $p<<0.001[1x10^{-15}]$) and 27% in women ((5%-54%), $p=0.012$).

Sensitivity analyses

After exclusion of subjects with hyperP, P was associated with CAC in both men (β:0.46 (0.32-0.60), $p<<0.001$ [$p=2x10^{-10}$], n=876) and women (β:0.17 (0.01-0.33), $p=0.032$, n=979). In men, results from Model II showed slight attenuation (men: β:0.40 (0.27-0.53), $p<<0.001$ [$p=1x10^{-8}$]). Exclusion of CKD and hyperP yielded [men β:0.44 (0.28-0.59), $p<<0.001$ [$p=5x10^{-8}$], n=766; women β:0.17 (0.001-0.33), $p=0.049$, n=846]. These results were slightly attenuated in men after adjustments for Model II (men β:0.38 (0.23-0.52), $p<<0.001$ [$p=1x10^{-6}$]). Furthermore, exclusion of CKD, hyperP and prevalent CVD showed [men: β:0.49 (0.32-0.66), $p<<0.001$ [$p=2x10^{-8}$], n=592; women: β:0.18 (0.004-0.35), $p=0.045$, n=766]. Adjustments for Model II yielded [men: β:0.46 (0.29-0.63), $p<<0.001$ [$p=1x10^{-7}$]; women: β:0.19 (0.01-0.36), $p=0.033$].

We found no evidence of effect modification by 25OHD (data not shown).

Results from Mendelian Randomization analyses

No evidence of departure from HWE was noticed in genotyped SNPs; for imputed SNPs, the quality (R^2) was above 96%. No frequency/strand inconsistency between the original GWAS (31) and RS was detected for the only palindromic SNP (rs2970818; A/T); therefore it was included in the score (**Supplementary Table 2**).

Concerning MR first assumption, the strength of the instruments was tested

regressing P levels (expressed in *entire units of mmol/L*) on the SNPs, stratified for all subjects with P levels available and further restricted to those with P and CAC (**Supplementary Table 3**). All 5 SNPs from CHARGE showed a significant association with P in the whole cohort with P available - though one SNP was not associated when further restricting to P & CAC available. Three SNPs from BBJ showed a significant association with P in the whole cohort but further restriction to subjects with P & CAC decreased power, reflected in F-statistics. Therefore, we first built a score including all 8 SNPs and subsequently applied sensitivity analyses.

Concerning MR second assumption, we regressed potential confounders on P scores: the 8-SNP score and the 5-SNP EUR-score (**Supplementary Table 4**). We found no association of the scores with total calcium, 25OHD, CRP, total cholesterol:HDL ratio and glucose; nor with BMI, smoking, prevalent CVD or DM.

Allele score analyses

Table 5 shows the results of the two stage least square regression of P - instrumented through the unweighted and weighted allelic score - and CAC adjusted for age, sex and 10 principal components in the whole cohort. Allele score has been scaled to be associated to 1 SD of P (0.14 mmol/L=0.43 mg/dL). A significant relation was found between P - instrumented through the unweighted score - and CAC (β:0.82 (0.04-1.59), p=0.039). Results for the weighted score were similar (β:0.81 (0.05-1.58), p=0.036).

Sex-stratified analysis suggested more consistency in the association between instrumented P and CAC in men than in women through both unweighted score [men β: 1.15 (-0.04-2.35), p=0.058, n=782; women β: 0.50 (-0.55-1.54), p=0.354, n=911] and weighted score [men β: 1.21 (0.005-2.42), p=0.049; women β: 0.45 (-0.56-1.46), p=0.379].

When we applied the leave-one-out SNP approach (from the score), we found that results lost significance after extracting **rs1697421** (β:0.42 (-0.45-1.28), p=0.344); **rs2970818** (β:0.84 (-0.01-1.69), p=0.054) and **rs35186465** (β:0.75 (-0.05-1.55), p=0.068), one at-a-time (**Table 5**).

Stratified analysis according to P levels, kidney function and CVD

P - instrumented through the unweighted score - remained associated to CAC after exclusion of hyperP (β:0.97 (0.05-1.90), p=0.039, n=1664), after exclusion of hyperP and CKD (β:1.23 (0.30-2.15), p=0.009, n=1446) and after exclusion of hyperP, CKD and prevalent CVD (β: 1.55 (0.45-2.65), p=0.006, n=1220) (**Table 6**). Results for the weighted score were similar. The implementation of robust standard errors did not change results (**Supplementary Table 5**).

Sensitivity analyses

Joint instruments analyses and Sargan statistics

P - instrumented through all 8 SNPs simultaneously - (**Supplementary Table 6**) was related to CAC (β:0.71 (0.04-1.39), p=0.038). The exclusion of one SNP at-a-time from the joint instruments yielded the same inference as its exclusion from the score: P is not related to CAC if the following SNPs are excluded, one at a time: **rs1697421**, **rs2970818** and **rs35186465**.

The Sargan tests could not reject the null hypothesis in any case, providing validity of the instruments and an indirect evidence of low heterogeneity among them.

EUR-score

Supplementary Table 7 shows that restriction to EUR-score did not attenuate the results of instrumented P and CAC (β:0.91 (0.05-1.77), p=0.039, n=1693). Results remained significant after excluding hyperP (β:0.96 (0.04-1.88), p=0.041, n=1664),

after excluding hyperP and CKD (β:1.25 (0.30-2.20), p=0.010, n=1446), and after excluding hyperP, CKD and prevalent CVD (β:1.30 (0.25-2.35), p=0.015, n=1220)

Assessment of potential horizontal pleiotropy

Concerning MR third assumption (**Supplementary Table 8**), from a frequentist approach the adaptive lasso regression found no evidence of invalidity of instruments; therefore the inference was similar as that obtained from *tsls*. There was no rejection of the Hansen test, meaning that all SNPs are valid and uncorrelated with error terms.

From a Bayesian approach we applied a method that incorporates pleiotropic effects into a fraction of SNPs and tests whether the association between instrumented exposure and outcome still remains. Allowing 49% of SNPs to exert pleiotropic effects and assuring model convergence, we found that instrumented P was still related to CAC: [posterior mean: 0.82; 95% *credible* interval (0.47-1.26)].

Discussion

In this population-based analyses serum P was strongly related to CAC - composed mostly of intimal calcification - even after excluding subjects with hyperP, CKD and prevalent CVD. It has been previously shown that hyperP or supraphysiological high P medium is able to induce an osteoblastic transformation of vascular smooth muscle cells with subsequent medial calcification (15, 26), characteristic of CKD. Nevertheless, whether normal P is related to intimal calcification has been much less explored (18, 19). Our data demonstrated an important sex-modification in this association, adding to sex differences previously found concerning P and all-cause mortality (42), CVD mortality (13) and atherosclerosis (14), where consistently a stronger (or unique) relation has been described in men.

When analyzed as a binary trait, P increases prevalence ratio (PR) in both sexes for CAC>100, considered of moderate risk, and in men P increases PR for CAC>300

and >400, considered high risk (2). Remarkably, P increases PR for CAC>1000 in both sexes, a category that confers a high mortality risk (27).

The implementation of MR methods where *instrumented* P is consistently associated with CAC strengthens the inference of our observational findings and supports causality. To the best of our knowledge, this is the first study to assess P and CAC relation through MR, by definition less prone to be affected by confounding and reverse causation. Of caution, an important source of bias in MR might be horizontal pleiotropy (43). Nevertheless, we applied elaborated regression models that assess (38) or allow pleiotropy (41): the persistence of similar results to standard *two stage least square* - through lasso regression - and the obtainment 18 of significant results despite allowing almost half of the SNPs to exert pleiotropic effects - through Bayesian modelling - confirm the robustness of our findings.

The association between P and CAC after restriction of MR to subjects without hyperP, CKD and prevalent CVD supports that increasing (but within normal range) P in the general population without clinical CVD is a pathogenic factor in CAC and challenges the concept that only severe hyperP - in the uremic context of CKD - is related to CAC. More importantly, it might provide an explanation for the emerging epidemiologic associations of P and increased mortality and CV events in cohorts (42) with mostly normal P; and to CVD mortality and atherosclerosis in men with strict normal P (13, 14). If these associations are causal there must be an underlying mechanism. CAC induction by an increasing - yet normal - P may be one of such mechanisms.

The approach of leaving-one-out SNP has recently been acknowledged as a robust penalization method to test validity in MR (39). We found that results were not significant when specific SNPs were omitted from the score, one-at-a-time:

a)rs1697421: its omission induces not only statistical significance loss but also abolishment of magnitude of results. This SNP is intergenic but its positional

candidate gene is *ALPL*, which encodes for tissue-nonspecific alkaline phosphatase (ALP). ALP was not related to CAC in our population study; but, remarkably, it hydrolyzes pyrophosphate (PPi) into P. PPi is one of the most potent calcification inhibitors (29). The condition where a SNP affects the outcome through a pathway affected by the risk factor of interest is termed vertical pleiotropy and does not invalidate MR findings. If this SNP influences ALP and its downstream activity/levels, PPi and P, it will correspond to *mediation* of the effect (43).

b)rs2970818: this SNP is also intergenic but one of the positional candidate genes is *FGF23*, which encodes for a key hormone in P homeostasis through increased renal P excretion (17). In contrast to observational studies linking higher FGF23 levels to arterial calcification, research at the cellular level has shown (16, 17) that FGF23 inhibits osteoblastic differentiation of vascular cells - partially through α-Klotho actions. Therefore, horizontal pleiotropy is unlikely.

c)rs35186465: To date, there are no know P-related genes annotated to this SNP.

Therefore, the association of instrumented P with CAC is explained mostly by the contribution of three SNPs from the allele score located in chromosomes 1, 12 and 17. Though a role from several SNPs located throughout the genome improves the validity from MR (39), it seems that **rs1697421** - near *ALPL* - plays a key role.

Besides FGF23 and α-Klotho, P is regulated by 1,25-dihydroxyvitamin D and parathyroid hormone (PTH) levels; both positively related to CAC. The significant results from MR analyses decrease their likelihood as confounders. Nevertheless, as PTH has been related to arterial calcification even at normal levels and PTH increases with increased P, our data cannot rule out a role of PTH on CAC.

Two main pathways of P-induced calcification have been described in the coronary bed: a) a passive deposition of calcium and P, strongly regulated by ALP-PPi-P, and b) an active process of *osteoblastic differentiation* of vascular pericytes and calcifying vascular cells, able to synthesize matrix vesicles, which start the

mineralization process. Current evidence has shown that ALP, PPi and P are present in matrix vesicles surfaces of atherosclerotic plaques (4), linking closely both mechanisms of calcification in CAC and potentially providing a biological explanation for our results.

We found an apparent dose-effect relation in P and CAC, with *normal* P thresholds of 1.09 mmol/L (3.35 mg/dL) in men and 1.37 mmol/L (4.24 mg/dL) in women. Interestingly, Dhingra et al (Framingham study) described a close cut-off for P of 3.5 mg/dL (1.13 mmol/L) above which CVD mortality and morbidity increased (12). The authors stated that it was not clear whether increasing P within normal range was associated with CVD risk - our data are able to answer this question in a confirmatory way.

We also found that *normal* levels of the calcium*phosphate product were related to CAC. Recent literature highlights that circulating calciprotein particles, composed of calcium, P and calcification inhibitors such as fetuin A, are crucial in calcification and that their compositions dictate whether pathologic mineralization is inhibited or not (44). The calcium*phosphate product within calciprotein particles has been identified as the culprit in this process. Similar to P, it might be that a normal product in serum does not reflect a safe product at the cellular level.

The stronger associations observed in men are consistent to previous research on P and CVD [atherosclerosis (14), CV event rates (42) and CVD mortality (13, 42)]. These results are unexpected, especially because women have higher P and because the protective effect of 17β-estradiol in arterial calcification (45) is predominant in premenopausal women. We can only speculate whether the association between calcium and phosphate levels plays a role in the sex difference as we (and others) have found an inverse relation between P and ionized calcium in women but not in men, and an inverse relation between them seems necessary to keep a constant calcium*phosphate product in serum (46, 47).

It is important to add that P intake has also been related to arterial calcification,

as an abrupt postprandial P increase suffices to initiate mineralization within seconds and to decrease *Klotho* expression (48).

This study has several limitations. We had a small sample size and no measurements on 1,25 OH-vitamin D, FGF23 and PTH levels, nor information on P intake. CAC and ALP were not determined simultaneously. Several tests were not suitable due to our one-sample MR. But there are several strengths, especially concerning the results from MR, that provides a formal test of causality provided the assumptions are fulfilled. Results from F-statistics strongly suggest that our findings are not affected by weak-instrument bias. We were able to perform important stratified analyses and to test instruments validity.

To conclude, we hereby provide both frequentist and Bayesian evidence from MR approach that normal P is a causative factor in CAC in the general population without hyperP, without prevalent CVD, and with normal kidney function. We add more evidence to support the concept of phosphotoxicity (9) and our results call for a review of the current normal P range. We agree with the European Food and Safety Agency (49) that more research is needed to study the relationships between dietary intake of P and serum P levels and adverse health outcomes. Public health policies might be needed to decrease P intake, due to the growing evidence of P as a continuous risk factor for adverse outcomes such as atherosclerosis, CVD mortality and now, CAC. Further research should focus in unveiling the underlying mechanisms of the detrimental effects of P in human health and to establish a threshold above which P must be considered harmful (50), especially because a large fraction of the population appears to be exposed to non-safe P levels.

Acknowledgments

The authors are in debt to the important expert advice that Prof. Stephen Burgess and Prof. George Davey Smith provided for the analyses of this dataset. Their help and kindness are highly appreciated.

Sources of funding

The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The funding sources had no influence in the study design, collection, analysis, interpretation of data, writing of the report and in the decision to submit the article.

Disclosures

Authors declare that they have no conflict of interest.

Table 1. General characteristics of subjects in RS-I with coronary artery calcification information available according to quintiles of fasting phosphate levels

	Men						Women					
	Phosphate in quintiles						**Phosphate in quintiles**					
	1	**2**	**3**	**4**	**5**	***p****	**1**	**2**	**3**	**4**	**5**	***p****
I) RS-I												
N	175	176	175	176	176		202	202	202	202	203	
(**P in mmol/L**)	(0.83)	(0.95)	(1.02)	(1.09)	(1.21)		(0.98)	(1.10)	(1.17)	(1.24)	(1.37)	
Age (y)	70.7	71.2	70.6	70.6	70.5	0.475	70.7	70.3	71.1	70.8	70.6	0.797
BMI (kg/m^2)	26.8	26.6	26.4	26.6	26.2	0.072	29.1	27.6	27.3	26.9	26.3	**<0.001**
Ever smoke (%)	93.1	89.1	93.1	94.3	94.3	0.223	49.2	51.5	55.9	57.7	53.5	0.189
Systolic BP (mm Hg)	145.4	142.8	144.7	145.1	143.7	0.913	141.7	143.9	143.6	139.3	142.5	0.514
Diastolic BP (mm Hg)	77.0	76.1	77.7	78.3	76.2	0.934	73.9	75.8	74.7	74.0	74.3	0.727
Ionized Ca^{++} (mmol/L)†	1.29	1.30	1.29	1.29	1.28	0.064	1.31	1.30	1.29	1.30	1.28	**<0.001**
Calcium (mmol/L)	2.39	2.41	2.40	2.40	2.42	**0.018**	2.43	2.44	2.44	2.44	2.46	**0.003**
Ca*P product (mmol^2/L^2)	1.98	2.28	2.44	2.61	2.94	**<0.001**	2.37	2.69	2.86	3.04	3.38	**<0.001**
ALP (U/L)†	79.5	76.1	75.5	75.1	74.8	0.100	80.1	77.1	77.8	79.8	83.1	0.436
25(OH)D (nmol/L)	66.5	63.1	65.9	59.6	61.5	**0.016**	49.2	51.5	49.0	49.2	50.5	0.897
CAC score	468.5	652.1	710.3	788.2	1093.2	**<0.001**	239.9	197.6	309.1	341.5	280.0	0.085
CRP (mg/L)	3.98	3.48	3.59	3.60	5.23	0.050	4.79	3.75	3.57	3.32	3.09	**0.007**
Glucose (mmol/L)	6.10	5.97	6.22	6.02	6.04	0.870	6.12	5.77	5.79	5.77	5.64	**0.001**
Creatinine (µmol/L)	92.0	90.6	89.9	91.0	95.4	0.402	71.7	71.5	72.5	71.8	70.8	0.498
eGFR (mL/min)	74.2	74.0	75.2	75.2	76.1	0.179	74.7	75.3	73.8	74.7	75.8	0.511

Chol to HDL ratio	4.76	4.91	4.80	4.66	4.42	**0.002**	4.42	4.41	4.32	4.22	4.10	**0.004**
Prevalent CVD (%)	19.6	25.9	23.7	25.0	23.0	0.444	7.07	8.21	5.85	9.84	8.00	0.563
Prevalent DM (%)	12.6	13.6	14.9	15.3	15.9	0.285	17.3	10.4	13.9	9.90	8.37	**0.011**

* *p* values correspond to age-adjusted significance of trend across quintiles. BMI: body mass index; BP: blood pressure; ionized Ca++: ionized calcium levels; calcium: total calcium levels; Ca*P product: total calcium x phosphate levels; ALP: alkaline phosphatase levels; 25(OH)D: 25-hydroxyvitamin D levels; CRP: C-reactive protein; prevalent DM: prevalent diabetes mellitus; eGFR: estimated glomerular filtration rate; Chol to HDL ratio: total cholesterol to HDL cholesterol ratio; prevalent CVD: prevalent cardiovascular disease

† Ionized calcium and alkaline phosphatase levels were not assessed simultaneously to P levels

Table 2a. Serum phosphate levels and coronary artery calcification scores in RS-I

	Model I			Model II		
	n	***β* (95% CI)***	***p***	**n**	***β* (95% CI)***	***p***
Men	878	**0.45** (0.32 to 0.58)	<0.001	878	**0.38** (0.26 to 0.51)	<0.001
Women	1011	**0.18** (0.04 to 0.32)	0.010	1011	**0.20** (0.06 to 0.34)	0.005
Total	1889	**0.31** (0.21 to 0.41)	<0.001	1889	**0.29** (0.19 to 0.38)	<0.001

* *β*s were obtained from generalized linear models and expressed per SD increase in phosphate (0.14 mmol/L=0.43 mg/dL)

Model I: adjusted for age, BMI, smoking

Model II: adjusted for age, BMI, blood pressure, smoking, prevalent cardiovascular disease, prevalent diabetes mellitus and serum levels of 25-hydroxyvitamin D, total calcium, CRP, total cholesterol to HDL cholesterol ratio and glucose

Table 2b. Serum phosphate levels and coronary artery calcification scores in RS-I stratified by eGFR

	eGFR≥60 mL/min*			**eGFR<60 mL/min***		
	n	***β* (95% CI)†**	***p***	**n**	***β* (95% CI)†**	***p***
Model I						
Men	768	**0.43** (0.28 to 0.59)	<0.001	110	**0.52** (0.29 to 0.74)	<0.001
Women	877	**0.18** (0.03 to 0.33)	0.020	134	0.17 (-0.23 to 0.58)	0.397
Total	1645	**0.29** (0.19 to 0.40)	<0.001	244	**0.38** (0.17 to 0.60)	0.001
Model II						
Men	768	**0.38** (0.23 to 0.52)	<0.001	110	**0.39** (0.16 to 0.63)	0.001
Women	877	**0.19** (0.04 to 0.34)	0.013	134	0.22 (-0.19 to 0.63)	0.297
Total	1645	**0.28** (0.17 to 0.38)	<0.001	244	**0.33** (0.11 to 0.54)	0.003

* eGFR estimated from creatinine-based Chronic Kidney Disease Epidemiology Collaboration equations

† *β*s were obtained from generalized linear models and expressed per SD increase in phosphate (0.14 mmol/L=0.43 mg/dL)

Model I: adjusted for age, BMI, smoking

Model II: adjusted for age, BMI, blood pressure, smoking, prevalent cardiovascular disease, prevalent diabetes mellitus and serum levels of 25-hydroxyvitamin D, total calcium, CRP, total cholesterol to HDL cholesterol ratio and glucose

Table 3. Serum phosphate levels in quintiles and coronary artery calcification scores in RS-I

	Men			Women			
n	**P levels mean (range)***	***β* (95% CI)†,‡**	***p***	**n**	**P levels mean (range)***	***β* (95% CI)†,‡**	***p***
175	0.83 (0.63-0.91)	1 (Ref)		202	0.98 (0.74-1.06)	1 (Ref)	
176	0.95 (0.91-0.98)	0.25 (-0.15 to 0.66)	0.223	202	1.10 (1.06-1.14)	0.02 (-0.42 to 0.45)	0.935
175	1.02 (0.98-1.05)	0.30 (-0.11 to 0.71)	0.151	202	1.17 (1.14-1.20)	0.03 (-0.41 to 0.46)	0.903
176	1.09 (1.05-1.13)	**0.80** (0.39 to 1.21)	<0.001	202	1.24 (1.20-1.28)	0.24 (-0.20 to 0.68)	0.282
176	1.21 (1.13-2.47)	**1.09** (0.68 to 1.50)	<0.001	203	1.37 (1.28-1.70)	**0.61** (0.17 to 1.05)	0.007
p_{trend}		<< 0.001				0.004	

* Phosphate quintiles are expressed in mmol/L

† *β*s were obtained from generalized linear models. First quintile of phosphate was set as reference

‡ Analyses were adjusted for age, BMI and smoking

Table 4. Serum phosphate x calcium product levels in quintiles and coronary artery calcification scores in RS-I

	Men			Women			
n	**Product** mean (range)*	***β* (95% CI)**[†,‡]	***p***	**n**	**Product** mean (range)*	***β* (95% CI)**[†,‡]	***p***
175	1.97 (1.50-2.16)	1 (Ref)		202	2.35 (1.67-2.57)	1 (Ref)	
176	2.27 (2.16-2.36)	0.03 (-0.38 to 0.43)	0.888	202	2.68 (2.58-2.77)	-0.03 (-0.47 to 0.40)	0.881
175	2.44 (2.36-2.52)	**0.48** (0.08 to 0.89)	0.020	202	2.86 (2.77-2.96)	0.30 (-0.13 to 0.73)	0.177
176	2.62 (2.52-2.71)	**0.76** (0.35 to 1.16)	<0.001	202	3.05 (2.96-3.15)	**0.47** (0.03 to 0.90)	0.035
176	2.96 (2.71-6.57)	**1.15** (0.74 to 1.56)	<0.001	203	3.40 (3.16-4.20)	**0.60** (0.16 to 1.04)	0.008
p_{trend}		<< 0.001				0.001	

* Calcium*phosphate product levels are expressed in $mmol^2/L^2$

[†] *β*s were obtained from generalized linear models. First quintile of calcium*phosphate product level was set as reference

[‡] Analyses were adjusted for age, BMI and smoking

Table 5. Mendelian Randomization results for serum P and CAC in RS-I: allelic score method applied to whole cohort

	Unweighted instruments			Weighted instruments*		
	n	***β* (95% CI)**[†,‡]	***p***	**n**	***β* (95% CI)**[†,‡]	***p***
Allele score	1693	**0.82** (0.04 to 1.59)	0.039	1693	**0.81** (0.05-1.58)	0.036
Leaving out[§]:						
rs1697421 (*ALPL*)	1693	0.42 (-0.45 to 1.28)	0.344	1693	0.38 (-0.47 to 1.23)	0.380
rs17265703 (*CASR*)	1693	**0.81** (0.07 to 1.55)	0.033	1693	**0.81** (0.07 to 1.54)	0.031
rs9469578	1693	**0.88** (0.05 to 1.72)	0.038	1693	**0.90** (0.07 to 1.73)	0.033
rs947583	1693	**1.04** (0.05 to 2.04)	0.040	1693	**1.00** (0.05 to 1.94)	0.038
rs2970818 (*FGF23*)	1693	0.84 (-0.01 to 1.69)	0.054	1693	0.84 (-0.001 to 1.68)	0.050
rs17060705 (*ENPP3*)	1693	**0.96** (0.14 to 1.77)	0.022	1693	**0.98** (0.18 to 1.79)	0.016
rs35186465	1693	0.75 (-0.05 to 1.55)	0.068	1693	0.75 (-0.04 to 1.53)	0.063
rs178710 (*PHEX*)	1693	**0.81** (0.02 to 1.60)	0.045	1693	**0.81** (0.02 to 1.59)	0.043

***** Weights were derived from CHARGE meta-analysis and BioBank Japan Project. Internal weights were avoided.

[†] *β*s were derived from two stage least square for the score as a single instrument and adjusted for age, sex and 10 PCs

[‡] Standard interpretation: results expressed as change in outcome per SD increase in phosphate (0.14 mmol/l=0.43 mg/dL)

[§] Results from allelic score analyses with the subtraction of one SNP at-a-time. Closest annotated gene is displayed if known to be associated with (or possible related to) P homeostasis

Table 6. Mendelian Randomization results for serum P and CAC in RS-I: allelic score method applied in stratified analyses according to P levels, kidney function and prevalent cardiovascular disease.

	Unweighted instruments			Weighted instruments*		
	n	***β* (95% CI)**[†,‡]	***p***	**n**	***β* (95% CI)**[†,‡]	***p***
Whole cohort						
Allele score	1693	**0.82** (0.04 to 1.59)	0.039	1693	**0.81** (0.05 to 1.58)	0.036
Stratified analyses						
Excluding hyperP[§]	1664	**0.97** (0.05 to 1.90)	0.039	1664	**0.98** (0.06 to 1.91)	0.037
Excluding CKD[‖]	1470	**1.04** (0.21 to 1.86)	0.014	1470	**1.02** (0.21 to 1.82)	0.013
Excluding hyperP & CKD	1446	**1.23** (0.30 to 2.15)	0.009	1446	**1.22** (0.30 to 2.13)	0.009
Excluding prevalent CVD[#]	1397	**1.21** (0.27 to 2.15)	0.012	1397	**1.16** (0.26 to 2.07)	0.012
Excluding hyperP, CKD and prevalent CVD	1220	**1.55** (0.45 to 2.65)	0.006	1220	**1.50** (0.43 to 2.57)	0.006

* Weights were derived from CHARGE meta-analysis and BioBank Japan Project. Internal weights were avoided.

† *β*s were derived from two stage least square for the score as a single instrument and adjusted for age, sex and 10 PCs

‡ Standard interpretation: results expressed as change in outcome per SD increase in phosphate (0.14 mmol/L=0.43 mg/dL)

§ HyperP: hyperphosphatemia, defined as a phosphate level >1.45 mmol/L (>4.5 mg/dL)

‖CKD: chronic kidney disease, defined as a glomerular filtration rate <60 mL/min

Prevalent CVD: prevalent cardiovascular disease, defined as prevalent myocardial infarction, revascularization, stroke and heart failure

REFERENCES

1. **London GM**. Mechanisms of arterial calcifications and consequences for cardiovascular function. *Kidney Int Suppl* (2011) 2013;3(5):442-445. doi: 10.1038/kisup.2013.92

2. **Budoff MJ, Achenbach S, Blumenthal RS, Carr JJ, Goldin JG, Greenland P, Guerci AD, Lima JAC, Rader DJ, Rubin GD, Shaw LJ, Wiegers SE; American Heart Association Committee on Cardiovascular Imaging and Intervention; American Heart Association Council on Cardiovascular Radiology and Intervention; American Heart Association Committee on Cardiac Imaging, Council on Clinical Cardiology.** Assessment of coronary artery disease by cardiac computed tomography: a scientific statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology. *Circulation* 2006;114(16):1761-1791. doi: 10.1161/CIRCULATIONAHA.106.178458

3. **Chen NX, Moe SM**. Vascular calcification: pathophysiology and risk factors. *Curr Hypertens Rep* 2012;14(3):228-237. doi: 10.1007/s11906-012-0265-8

4. **Ruiz JL, Weinbaum S, Aikawa E, Hutcheson JD**. Zooming in on the genesis of atherosclerotic plaque microcalcifications. *J Physiol* 2016;594(11):2915-2927. doi: 10.1113/JP271339

5. **Detrano R, Guerci AD, Carr JJ, Bild DE, Burke G, Folsom AR, Liu K, Shea S, Szklo M, Bluemke DA, O'Leary DH, Tracy R, Watson K, Wong ND, Kronmal RA**. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med* 2008;358(13):1336-1345. doi: 10.1056/NEJMoa072100

6. **Nakamura S, Ishibashi-Ueda H, Niizuma S, Yoshihara F, Horio T, Kawano Y**. Coronary calcification in patients with chronic kidney disease and coronary artery disease. *Clin J Am Soc Nephrol* 2009;4(12):1892- 1900. doi: 10.2215/CJN.04320709

7. **Greenland P, Blaha MJ, Budoff MJ, Erbel R, Watson KE**. Coronary Calcium Score and Cardiovascular Risk. *J Am Coll Cardiol* 2018;72(4):434-447.

doi: 10.1016/j.jacc.2018.05.027

8. **Ichikawa S, Imel EA, Kreiter ML, Yu X, Mackenzie DS, Sorenson AH, Goetz R, Mohammadi M, White EK, Econs MJ**. A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Clin Invest* 2007;117(9):2684-91. doi: 10.1172/JCI31330

9. **Ohnishi M, Razzaque MS**. Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. *FASEB J* 2010;24(9):3562-3571. doi: 10.1096/fj.09-152488

10. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK**. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998;31(4):607-617.

11. **Otsuka F, Sakakura K, Yahagi K, Joner M, Virmani R**. Has our understanding of calcification in human coronary atherosclerosis progressed? *Arterioscler Thromb Vasc Biol* 2014;34(4):724-736. doi:10.1161/ATVBAHA.113.302642

12. **Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB Sr, Gaziano JM, Vasan RS**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007;167(9):879-885. doi: 10.1001/archinte.167.9.879

13. **Campos-Obando N, Lahousse L, Brusselle G, Stricker BH, Hofman A, Franco OH, Uitterlinden AG, Zillikens MC**. Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men. *Eur J Epidemiol* 2018; 33(9): 859-871. doi: 10.1007/s10654-018-0407-7.

14. **Onufrak SJ, Bellasi A, Shaw LJ, Herzog CA, Cardarelli F, Wilson PW, Vaccarino V, Raggi P**. Phosphorus levels are associated with subclinical atherosclerosis in the general population. *Atherosclerosis* 2008;199(2):424-431. doi: 10.1016/j.atherosclerosis.2007.11.004

15. **Villa-Bellosta R, Millan A, Sorribas V**. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am J Physiol Cell Physiol* 2011;300(1):C210-220. doi: 10.1152/ajpcell.00229.2010

16. **Nakahara T, Kawai-Kowase K, Matsui H, Sunaga H, Utsugi T, Iso T, Arai M, Tomono S, Kurabayashi M**. Fibroblast growth factor 23 inhibits osteo-

blastic gene expression and induces osteoprotegerin in vascular smooth muscle cells. *Atherosclerosis* 2016;253:102-110. doi: 10.1016/j.atherosclerosis.2016.08.010

17. **Scialla JJ, Lau WL, Reilly MP, Isakova T, Yang HY, Crouthamel MH, Chavkin NW, Rahman M, Wahl P, Amaral AP, Hamano T, Master SR, Nessel L, Chai B, Xie D, Kallem RR, Chen J, Lash JP, Kusek JW, Budoff MJ, Giachelli CM, Wolf M; Chronic Renal Insufficiency Cohort Study Investigators**. Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. *Kidney Int* 2013;83(6):1159-1168. doi: 10.1038/ki.2013.3

18. **Shin S, Kim KJ, Chang HJ, Cho I, Kim YJ, Choi BW, Rhee Y, Lim SK, Yang WI, Shim CY, Ha JW, Jang Y, Chung N**. Impact of serum calcium and phosphate on coronary atherosclerosis detected by cardiac computed tomography. *Eur Heart J* 2012;33(22):2873-2881. doi: 10.1093/eurheartj/ehs152

19. **Grønhøj MH, Gerke O, Mickley H, Steffensen FH, Lambrechtsen J, Sand NPR, Rasmussen LM, Olsen MH, Diederichsen A**. Associations between calcium-phosphate metabolism and coronary artery calcification; a cross sectional study of a middle-aged general population. *Atherosclerosis* 2016;251:101-108. doi: 10.1016/j.atherosclerosis.2016.06.001

20. **Burgess S, Small DS, Thompson SG**. A review of instrumental variable estimators for Mendelian randomization. *Stat Methods Med Res* 2017;26(5):2333-2355. doi: 10.1177/0962280215597579

21. **Ikram MA, Brusselle GGO, Murad SD, van Duijn CM, Franco OH, Goedegebure A, Klaver CCW, Nijsten TEC, Peeters RP, Stricker BH, Tiemeier H, Uitterlinden AG, Vernooij MW, Hofman A**. The Rotterdam Study: 2018 update on objectives, design and main results. *Eur J Epidemiol* 2017;32(9):807-850. doi: 10.1007/s10654-017-0321-4

22. **Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW**. The Rotterdam Study: 2016 objectives and design update. *Eur J Epidemiol* 2015;30(8):661-708. doi: 10.1007/s10654-015-0082-x

23. **Koehler EM, Sanna D, Hansen BE, van Rooij FJ, Heeringa J, Hofman A, Tiemeier H, Stricker BH, Schouten JN, Janssen HL**. Serum liver en-

zymes are associated with all-cause mortality in an elderly population. *Liver Int* 2014;34(2):296-304. doi: 10.1111/liv.12311

24. **Leening MJ, Kavousi M, Heeringa J, van Rooij FJ, Verkroost-van Heemst J, Deckers JW, Mattace9 Raso FU, Ziere G, Hofman A, Stricker BH, Witteman JC**. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. *Eur J Epidemiol* 2012;27(3):173-185. doi: 10.1007/s10654-012-9668-8

25. **Brahimaj A, Muka T, Kavousi M, Laven JS, Dehghan A, Franco OH**. Serum dehydroepiandrosterone levels are associated with lower risk of type 2 diabetes: the Rotterdam Study. *Diabetologia* 2017;60(1):98- 106. doi: 10.1007/s00125-016-4136-8

26. **Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM**. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res* 2011;109(6):697-711. doi: 10.1161/CIRCRESAHA.110.234914.

27. **Shaw LJ, Giambrone AE, Blaha MJ, Knapper JT, Berman DS, Bellam N, Quyyumi A, Budoff MJ, Callister TQ, Min JK**. Long-Term Prognosis After Coronary Artery Calcification Testing in Asymptomatic Patients: A Cohort Study. *Ann Intern Med* 2015;163(1):14-21. doi: 10.7326/M14-0612

28. **Sohl E, de Jongh RT, Heymans MW, van Schoor NM, Lips P**. Thresholds for Serum 25(OH)D Concentrations With Respect to Different Outcomes. *J Clin Endocrinol Metab* 2015;100(6):2480-2488. doi: 10.1210/jc.2015-1353

29. **Haarhaus M, Brandenburg V, Kalantar-Zadeh K, Stenvinkel P, Magnusson P**. Alkaline phosphatase: a novel treatment target for cardiovascular disease in CKD. *Nat Rev Nephrol* 2017;13(7):429-442. doi: 10.1038/nrneph.2017.60

30. **Burgess S, Thompson SG**. Use of allele scores as instrumental variables for Mendelian randomization. *Int J Epidemiol* 2013;42(4):1134-1144. doi: 10.1093/ije/dyt093

31. **Kestenbaum B, Glazer NL, Kottgen A, Felix JF, Hwang SJ, Liu Y, Lohman K, Kritchevsky SB, Hausman DB, Petersen AK, Gieger C, Ried JS, Meitinger T, Strom TM, Wichmann HE, Campbell H, Hayward C, Rudan I, de Boer IH, Psaty BM, Rice KM, Chen YD, Li M, Arking DE, Boerwinkle E, Coresh J, Yang Q, Levy D, van Rooij FJ, Dehghan A, Rivadeneira F, Uit-**

terlinden AG, Hofman A, van Duijn CM, Shlipak MG, Kao WH, Witteman JC, Siscovick DS, Fox CS. Common genetic variants associate with serum phosphorus concentration. *J Am Soc Nephrol* 2010;21(7):1223-1232. doi: 10.1681/ASN.2009111104

32. **Kanai M, Akiyama M, Takahashi A, Matoba N, Momozawa Y, Ikeda M, Iwata N, Ikegawa S, Hirata M, Matsuda K, Kubo M, Okada Y, Kamatani Y**. Genetic analysis of quantitative traits in the Japanese population links cell types to complex human diseases. *Nat Genet* 2018;50(3):390-400. doi: 10.1038/s41588-018-0047-6

33. **Schork NJ, Murray SS, Frazer KA, Topol EJ**. Common vs. rare allele hypotheses for complex diseases. *Curr Opin Genet Dev* 2009;19(3):212-219. doi: 10.1016/j.gde.2009.04.010

34. **Iribarren C, Lu M, Jorgenson E, Martinez M, Lluis-Ganella C, Subirana I, Salas E, Elosua R**.Weighted Multi-marker Genetic Risk Scores for Incident Coronary Heart Disease among Individuals ofAfrican, Latino and East-Asian Ancestry. *Sci Rep* 2018;8(1):6853. doi: 10.1038/s41598-018-25128-x

35. **König IR, Loley C, Erdmann J, Ziegler A**. How to include chromosome X in your genome-wide association study. *Genet Epidemiol* 2014;38(2):97-103. doi: 10.1002/gepi.21782

36. **Didelez V, Sheehan N**. Mendelian randomization as an instrumental variable approach to causal inference. *Stat Methods Med Res* 2007;16(4):309-330. doi: 10.1177/0962280206077743

37. **Hartwig FP, Davies NM**. Why internal weights should be avoided (not only) in MR-Egger regression. *Int J Epidemiol* 2016;45(5):1676-1678. doi: 10.1093/ije/dyw240

38. **Windmeijer F, Farbmacher H, Davies N, Davey Smith G**. On the use of the lasso for instrumental variables estimation with some invalid instruments. *Journal of the American Statistical Association* 2018. doi: 10.1080/01621459.2018.1498346

39. **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

40. **Palmer TM, Lawlor DA, Harbord RM, Sheehan NA, Tobias JH, Timpson NJ, Davey Smith G, Sterne JA**. Using multiple genetic variants as instrumental variables for modifiable risk factors. *Stat Methods Med Res* 2012;21(3):223-242. doi: 10.1177/0962280210394459

41. **Berzuini C, Guo H, Burgess S, Bernardinelli L**. A Bayesian approach to Mendelian randomization with multiple pleiotropic variants. *Biostatistics* 2018. doi: 10.1093/biostatistics/kxy027

42. **Onufrak SJ, Bellasi A, Cardarelli F, Vaccarino V, Muntner P, Shaw LJ, Raggi P**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all cause mortality. *Am J Epidemiol* 2009;169(1):67-77. doi: 10.1093/aje/kwn285

43. **Hemani G, Bowden J, Davey Smith G**. Evaluating the potential role of pleiotropy in Mendelian randomization studies. *Hum Mol Genet* 2018;27(R2):R195-R208. doi: 10.1093/hmg/ddy163

44. **Viegas CSB, Santos L, Macedo AL, Matos AA, Silva AP, Neves PL, Staes A, Gevaert K, Morais R, Vermeer C, Schurgers L, Simes DC**. Chronic Kidney Disease Circulating Calciprotein Particles and Extracellular Vesicles Promote Vascular Calcification: A Role for GRP (Gla-Rich Protein). *Arterioscler Thromb Vasc Biol* 2018;38(3):575-587. doi: 10.1161/ATVBAHA.117.310578

45. **Rzewuska-Lech E, Jayachandran M, Fitzpatrick LA, Miller VM**. Differential effects of 17beta24 estradiol and raloxifene on VSMC phenotype and expression of osteoblast-associated proteins. *Am J Physiol Endocrinol Metab* 2005;289(1):E105-112. doi: 10.1152/ajpendo.00366.2004

46. **Felsenfeld AJ, Rodriguez M**. Phosphorus, regulation of plasma calcium, and secondary hyperparathyroidism: a hypothesis to integrate a historical and modern perspective. *J Am Soc Nephrol* 1999;10(4):878-890.

47. **Calvo MS, Kumar R, Heath H 3rd**. Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. *J Clin Endocrinol Metab* 1988;66(4):823-829. doi:10.1210/jcem-66-4-823

48. **Kuro-o M**. Klotho, phosphate and FGF-23 in ageing and disturbed mineral

metabolism. *Nat Rev Nephrol* 2013;9(11):650-660. doi: 10.1038/nrneph.2013.111

49. **European Food Safety Authority**. Assessment of one published review on health risks associated with phosphate additives in food. *EFSA Journal* 2013;11(1):3444-71. doi:10.2903/j.efsa.2013.3444

50. **Komaba H, Fukagawa M**. Phosphate-a poison for humans? *Kidney Int* 2016;90(4):753-763. doi:10.1016/j.kint.2016.03.039

Supplementary Material I

Supplementary Table 1. Serum phosphate levels and CAC prevalence ratios in RS-I stratified by CAC category according to Agatston's units

	Model I			Model II		
	n / total	**PR* (95% CI)**	***p***	**n / total**	**PR* (95% CI)**	***p***
CAC score >100						
Men	600/878	**1.09** (1.06 to 1.12)	<0.001	600/878	**1.07** (1.03 to 1.10)	<0.001
Women	418/1011	**1.07** (1.00 to 1.15)	0.043	418/1011	**1.07** (1.00 to 1.14)	0.049
CAC score >300						
Men	444/878	**1.11** (1.07 to 1.16)	<0.001	444/878	**1.08** (1.03 to 1.13)	0.001
Women	237/1011	1.11 (0.99 to 1.24)	0.054	237/1011	1.10 (0.99 to 1.21)	0.051
CAC score >400						
Men	398/878	**1.13** (1.08 to 1.18)	<<0.001	398/878	**1.08** (1.03 to 1.13)	0.001
Women	199/1011	1.10 (0.97 to 1.24)	0.139	199/1011	1.07 (0.96 to 1.19)	0.241
CAC score >1000						
Men	206/878	**1.19** (1.14 to 1.25)	<<0.001	206/878	**1.16** (1.09 to 1.24)	<0.001
Women	70/1011	**1.27** (1.05 to 1.54)	0.012	70/1011	**1.37** (1.14 to 1.65)	0.001

***** PR reflects change in CAC prevalence ratio per SD increase in phosphate (0.14 mmol/l=0.43 mg/dL)

Model I: adjusted for age, BMI and smoking

Model II: adjusted for age, BMI, blood pressure, smoking, prevalent cardiovascular disease, prevalent diabetes mellitus and serum levels of 25-hydroxyvitamin D, total calcium, CRP, total cholesterol to HDL cholesterol ratio and glucose

Supplementary Table 2. Characteristics of GWAS-significant SNPs for serum P score in RS-I derived from CHARGE GWAS meta-analysis and BioBank Japan Project

Genetic instruments for serum phosphate score								
Genotyped SNPs								
rsID	**Chr**	**Pos (Build 37)**	**EA***	**OA**	**EAF**	***β*†**	**HWE*p***	**Comments**
rs1697421	1	21823292	A	G	0.51	0.05	0.614	No departure from HWE
rs947583	6	136133659	C	T	0.29	0.04	0.189	No departure from HWE
Imputed autosomal SNPs								
rsID	**Chr**	**Pos (Build 37)**	**EA***	**OA**	**EAF**	***β*†**	**R^2**	**Comments**
rs17265703	3	122048644	A	G	0.86	0.04	0.99	Good imputation quality
rs9469578	6	33706479	C	T	0.92	0.06	0.99	Good imputation quality
rs17060705	6	132086493	A	G	0.08	0.06	0.99	Good imputation quality
rs2970818‡	12	4606168	A	T	0.08	0.05	0.97	Good imputation quality
rs35186465	17	66681582	A	G	0.25	0.04	0.99	Good imputation quality
Imputed X-linked SNP: men								
rs178710	X	22051034	A	G	0.54	0.04	0.99	Good imputation quality
Imputed X-linked SNP: women								
rs178710	X	22051034	A	G	0.54	0.04	0.99	Good imputation quality

rsID: SNP unique identification Chr: chromosome Pos: position EA: effect allele OA: other allele EAF: effect allele frequency HWE *p: p* value for Hardy Weinberg test within RS-I

R^2: imputation quality

* Effect alleles were aligned in an increasing P-levels sense

† βs: betas derived from GWAS meta-analysis or BioBank Japan Project. Internal weights were avoided

‡ Palindromic SNP with similar EA & EAF between CHARGE meta-analysis and RS-I, discarding strand inconsistencies

Supplementary Table 3. Test of MR assumption N°1: association of the unweighted genetic instruments with serum P levels in participants from RS-I, as assessed by *β, p* and F-statistic

	P levels available				P & CAC levels available			
Instrument	**n**	**F***	***β* (95% CI)†**	***p***	**n**	**F***	***β* (95% CI)†**	***p***
Genetic instruments derived from European ancestry study‡								
rs1697421	3491	470.4	0.01 (0.01-0.02)	$6x10^{-5}$	1693	263.0	0.01 (0.004-0.02)	0.006
rs17265703	3491	463.8	0.01 (0.003-0.02)	0.008	1693	258.1	-0.001(-0.01-0.01)	0.921
rs9469578	3491	462.6	0.01 (0.002-0.03)	0.023	1693	260.9	0.02 (0.001-0.04)	0.037
rs947583	3491	461.9	0.01 (0.002-0.01)	0.044	1693	265.2	0.02 (0.007-0.03)	0.001
rs2970818	3491	476.1	0.03 (0.02-0.04)	$6x10^{-7}$	1693	262.9	0.02 (0.007-0.04)	0.007
Genetic instruments derived from East Asian ancestry study§								
rs17060705	3491	465.7	0.02 (0.01-0.03)	0.001	1693	259.7	0.01 (-0.004-0.03)	0.122
rs35186465	3491	464.1	0.01 (0.003-0.02)	0.006	1693	259.4	0.01 (-0.003-0.02)	0.159
rs178710	3490	466.8	0.01 (0.003-0.01)	0.004	1693	260.4	0.01 (-0.004-0.01)	0.063
Allele score	3490	506.5	0.01 (0.009-0.01)	$1x10^{-16}$	1693	277.9	0.01 (0.007-0.01)	$1x10^{-7}$

* F: F-statistics, derived from sex-adjusted regressions of P on the genetic instrument

† *β*s derived from regression of P on the genetic instrument in RS-I, expressed as change in P levels (entire units of mmol/L) per increase in one risk allele in the SNP or in one unit in the allelic score. These *β*s were estimated with the unique purpose of testing MR assumption #1, not for weighting the allelic score for analyses

‡ GWAS-significant SNPs found in CHARGE meta-analysis

§ GWAS-significant SNPs found in BioBank Japan Project

Supplementary Table 4. Assessment of MR assumption N°2: association between unweighted allelic scores and potential confounders measured within RS-I

		08-SNP score*		EUR-score†	
Continuous outcomes					
n	**Potential confounder**	***β* (95% CI)**	***p***	***β* (95% CI)**	***p***
1693	BMI	0.02 (-1.38 to 1.41)	0.982	-0.41 (-1.97 to 1.15)	0.607
1693	T. calcium	-0.02 (-0.05 to 0.01)	0.268	-0.02 (-0.06 to 0.02)	0.368
1693	25OHD	1.21 (-7.15 to 9.56)	0.777	4.72 (-4.56 to 13.9)	0.319
1693	CRP	0.85 (-1.36 to 3.06)	0.449	0.08 (-2.38 to 2.55)	0.947
1693	Glucose	-0.23 (-0.76 to 0.30)	0.399	-0.46 (-1.05 to 0.13)	0.130
1693	Chol:HDL ratio	-0.36 (-0.82 to 0.11)	0.131	-0.33 (-0.86 to 0.18)	0.206
Categorical outcomes					
n	**Potential confounder**	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
1693	Smoking category	1.18 (0.59 to 2.41)	0.618	1.08 (0.49 to 2.38)	0.839
1693	Prevalent CVD	1.07 (0.38 to 2.96)	0.896	1.44 (0.45 to 4.62)	0.542
1693	Prevalent DM	0.55 (0.19 to 1.59)	0.269	0.59 (0.18 to 1.93)	0.385

* Unweighted scaled allele score derived from SNPs from BioBank Japan Project and CHARGE meta-analysis.

† Unweighted scaled allele score derived from SNPs from CHARGE meta-analysis. Results are age and sex-adjusted.

BMI: body mass index T. calcium: total calcium 25OHD: 25-hydroxyvitamin D CRP: C reactive protein Chol: HDL ratio: total cholesterol: HDL ratio Prevalent CVD: prevalent cardiovascular disease Prevalent DM: prevalent diabetes mellitus

Supplementary Table 5. Mendelian Randomization results for serum P and CAC in RS-I: allelic score method applied in stratified analyses according to P levels, kidney function and prevalent cardiovascular disease after implementation of robust standard errors

	Unweighted instruments			Weighted instruments*		
	n	*β* (95% CI)[†,‡]	*p*	n	*β* (95% CI)[†,‡]	*p*
Whole cohort						
Allele score	1693	**0.82** (0.06 to 1.57)	0.034	1693	**0.81** (0.07 to 1.55)	0.031
Stratified analyses						
Excluding hyperP[§]	1664	**0.97** (0.08 to 1.87)	0.033	1664	**0.98** (0.09 to 1.88)	0.031
Excluding CKD[‖]	1470	**1.04** (0.23 to 1.84)	0.011	1470	**1.02** (0.24 to 1.79)	0.010
Excluding hyperP & CKD	1446	**1.23** (0.33 to 2.12)	0.007	1446	**1.22** (0.34 to 2.10)	0.007
Excluding prevalent CVD[#]	1397	**1.21** (0.31 to 2.11)	0.008	1397	**1.16** (0.30 to 2.02)	0.008
Excluding hyperP, CKD and prevalent CVD	1220	**1.55** (0.51 to 2.59)	0.004	1220	**1.50** (0.48 to 2.51)	0.004

* Weights were derived from CHARGE meta-analysis and BioBank Japan Project. Internal weights were avoided.

† *βs* were derived from two stage least square for the score as a single instrument adjusted for age, sex and 10 PCs and after implementation of robust standard errors

‡ Standard interpretation: results expressed as change in outcome per SD increase in phosphate (0.14 mmol/l=0.43 mg/dL)

§ HyperP: hyperphosphatemia, defined as a phosphate level > 1.45 mmol/L (>4.5 mg/dL)

‖ CKD: chronic kidney disease, defined as a glomerular filtration rate < 60 mL/min

Prevalent CVD: prevalent cardiovascular disease, defined as prevalent myocardial infarction, revascularization, stroke and heart failure

Supplementary Table 6. Mendelian Randomization results for serum P and CAC in RS-I: joint instruments analyses method applied to whole cohort and tests of overidentification

		Unweighted instruments		Test of overidentification*	
	n	***β* (95% CI)†,‡**	***p***	**Sargan statistic§**	***Sargan p***
Joint instruments	1693	**0.71** (0.04 to 1.39)	0.038	χ^2_7: 6.65	0.465
Leaving out‖:					
rs1697421 (*ALPL*)	1693	0.34 (-0.40 to 1.08)	0.371	χ^2_6: 2.23	0.898
rs17265703 (*CASR*)	1693	**0.71** (0.04 to 1.39)	0.038	χ^2_6: 6.65	0.354
rs9469578	1693	**0.78** (0.06 to 1.51)	0.033	χ^2_6: 6.23	0.398
rs947583	1693	**0.97** (0.11 to 1.82)	0.026	χ^2_6: 5.26	0.511
rs2970818 (*FGF23*)	1693	0.74 (-0.01 to 1.49)	0.054	χ^2_6: 6.61	0.358
rs1706005 (*ENPP3*)	1693	**0.81** (0.11 to 1.50)	0.023	χ^2_6: 5.00	0.543
rs35186465	1693	0.67 (-0.03 to 1.36)	0.061	χ^2_6: 6.49	0.371
rs178710 (*PHEX*)	1693	**0.70** (0.01 to 1.40)	0.048	χ^2_6: 6.65	0.353

* Overidentification tests provide a measure of validity of all included instruments, where H_o states that included instruments are valid. Sargan is an overidentification test applied in one-sample MR setting

† *βs* derived from *tsls* regression with multiple instruments jointly and adjusted for age, sex and 10 PC

‡ Standard interpretation: results expressed as change in outcome per SD increase in phosphate (0.14 mmol/l=0.43 mg/dL)

§ Sargan statistic follows a χ^2_{j-1} d.f. distribution, where j is the number of genetic instruments

‖ MR test of joint instruments excluding one SNP at-a-time (penalization technique)

Supplementary Table 7. Mendelian Randomization results for serum P and CAC in RS-I: allelic score method including only European-ancestry derived instruments (EUR-score) applied in stratified analyses according to P levels and kidney function

		Unweighted EUR-score			Weighted EUR-score*	
	n	*β* (95% CI)[†,‡]	*p*	n	*β* (95% CI)[†,‡]	*p*
Whole cohort						
EUR ancestry allele score	1693	**0.91** (0.05 to 1.77)	0.039	1693	**0.95** (0.09 to 1.81)	0.030
Stratified analyses						
Excluding hyperP[§]	1664	**0.96** (0.04 to 1.88)	0.041	1664	**1.01** (0.08 to 1.94)	0.033
Excluding CKD[‖]	1470	**1.16** (0.25 to 2.07)	0.013	1470	**1.18** (0.29 to 2.07)	0.009
Excluding hyperP & CKD	1446	**1.25** (0.30 to 2.20)	0.010	1446	**1.28** (0.34 to 2.23)	0.008
Excluding prevalent CVD[#]	1397	**1.10** (0.06 to 2.14)	0.038	1397	**1.14** (0.14 to 2.14)	0.026
Excluding hyperP, CKD and prevalent CVD	1220	**1.30** (0.25 to 2.35)	0.015	1220	**1.32** (0.30 to 2.34)	0.011

* Weights were derived from CHARGE meta-analysis. Internal weights were avoided.

† *β*s were derived from *tsls* for the score as a single instrument and adjusted for age, sex and 10 PCs

‡ Standard interpretation: results expressed as change in outcome per SD increase in phosphate (0.14 mmol/L=0.43 mg/dL)

§ HyperP: hyperphosphatemia, defined as a phosphate level > 1.45 mmol/L (>4.5 mg/dL)

‖CKD: chronic kidney disease, defined as a glomerular filtration rate below 60 mL/min

Prevalent CVD: prevalent cardiovascular disease, defined as prevalent myocardial infarction, revascularization, stroke and heart failure

Supplementary Table 8. Assessment of potential horizontal pleiotropy through frequentist and Bayesian approaches

	Results	Comment
Frequentist approach		
Adaptive lasso*	Hansen test does not reject in the first place	No evidence of invalid instruments
Bayesian approach		
MCMC estimation†	Posterior mean: 0.82 (95% Credible Interval: 0.47-1.26)	Model achieved convergence

* The test first applies an overidentification test (Hansen test) to test the validity of instruments. In case of invalidity, it estimates a valid β provided less than 50% of instruments are invalid. Otherwise, *tsls* results are appropriate

† The Bayesian approach allows a subset (49%) of SNPs to be invalid due to pleiotropic effects and provides estimates in this condition. Output provides Markov Chain Monte Carlo derived posterior mean, its 95% Credible Interval and whether the model achieved convergence.

7

Influence of sex hormones on sexual dimorphism in calcium and phosphate homeostasis

Influence of sex hormones on sexual dimorphism in calcium and phosphate homeostasis

Authors:

Wera Nadia H. Koek*, Natalia Campos-Obando*, Bram C.J. van der Eerden, M. Arfan Ikram, André G. Uitterlinden, Johannes P.T.M. van Leeuwen and M. Carola Zillikens

*These authors contributed equally to this work

Status:

Manuscript ready for submission.

Abstract

Background: Sex differences in serum phosphate and calcium have been reported but the exact nature and underlying regulatory mechanisms remain unclear. We aimed to compare calcium and phosphate levels between sexes, and explore potential covariates to elucidate underlying mechanisms of sex differences in a prospective, population-based cohort study.

Methods: Pooled data of subjects >45 years from three independent cohorts of the Rotterdam Study (RS) were used: RS-I-3 (n=3623) RS-II-1 (n=2389), RS-III-1 (n=3241), with separate analyses from an additional time point of the first cohort RS-I-1 (n=1679). We selected subjects with availability of serum calcium, phosphate, kidney function (eGFR) at baseline and a subset with the availability of 25-hydroxyvitamin D (25(OH)D) and sex hormones and albumin, alkaline phosphatase (ALP) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).

Results: Compared to men, women had significantly higher total serum calcium and phosphate which was not explained by ALP, 25(OH)D or $1,25(OH)_2D_3$. Adjustments for serum testosterone but not for estradiol diminished sex differences in serum phosphate. Pre-menopausal women had higher serum phosphate but not calcium compared to men but lower compared to postmenopausal women. In the total group, both serum calcium and phosphate decreased with age with a significant interaction for sex differences for serum calcium but not phosphate.

Conclusion: Women >45 years have higher serum calcium and phosphate levels compared to men of similar age, not explained by vitamin D or ALP levels. Serum testosterone influences sex difference in serum phosphate but not calcium. Estradiol does not affect sex differences in serum calcium or phosphate.

Introduction

Calcium and phosphate are important electrolytes in human physiology. Calcium is one of the most abundant cations in the body. It is crucial for multiple metabolic processes such as neural transmission, blood coagulation and cell proliferation, and pivotal for bone mineralization.

Approximately 51% of total calcium is bound to proteins such as albumin and globulin while the remainder circulates in ionic form (free serum calcium or Ca^{2+}) [1]. Serum calcium levels are tightly controlled through the interaction of the intestines, kidneys, bone, and parathyroid glands. In hypocalcemia, parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are the major hormones controlling calcium homeostasis by stimulating calcium absorption in the intestines, calcium reabsorption in the kidney and bone resorption.

Phosphate is an important electrolyte in energy metabolism and is part of DNA and RNA structures [2]. Moreover, it is incorporated in extracellular matrix as hydroxyapatite during bone formation. Serum calcium and phosphate levels influence each other [1,2]. Serum phosphate is predominantly controlled through urinary excretion by the actions of PTH and the osteocyte-derived hormone fibroblast growth factor 23 (FGF23). Additionally, a marginal regulation of serum phosphate levels is exerted at the intestines through the action of $1,25(OH)_2D_3$ [3]. Serum phosphate is less tightly regulated than serum calcium. Phosphate is present in serum mainly in a free and ultra-filtrable form (85-90%), whilst the remainder (10-15%) is bound to proteins [2,4].

There have been several studies evaluating sex differences in serum calcium and/or phosphate. For serum calcium the results in these studies were less consistent than for serum phosphate but overall these studies found sex differences between post-menopausal women and men [5-8].Other studies evaluating serum calcium levels found differences between pre- and postmenopausal women, and between men and women at various ages but these results were either inconsistent or sex

differences were not systematically investigated in different age groups [9-12]. More consistent data are available showing that postmenopausal women have higher serum phosphate levels compared to men of similar age [9,13-17].

Serum calcium and phosphate imbalance has been linked to several disorders such as cardiovascular disease, metabolic syndrome, osteoporosis and mortality [5,16,18,19]. Besides, the calcium-phosphate product is associated with morbidity and mortality in patients with end-stage renal disease [19], although recent KDIGO guidelines suggest no additional value of this construct over individual serum levels of Ca and P in patients with CKD G3a–G5D (http://kdigo.org/wp90 content/uploads/2017/02/2017-KDIGO-CKD-MBD-GL-Update.pdf). Some of these conditions display marked sex-specific incidences [16,20]. Sex differences have also been shown for the accumulation of calcium and phosphate in coronary arteries [16]. Thus, it is possible that sex differences in the incidence of these diseases might be related to calcium and phosphate homeostasis and that the underlying mechanisms may have clinical consequences.

Therefore, in this study, we compared calcium and phosphate homeostasis between men and women in a population-based cohort study of elderly White Caucasians, i.e., the Rotterdam Study, and explored the role of potential confounders like vitamin D and sex hormones and how they influence calcium and phosphate homeostasis in both sexes.

Material and Methods

Rotterdam Study

The Rotterdam Study is a large prospective population-based cohort study of Caucasian subjects aged 45 years and older, living in the Ommoord district of Rotterdam, The Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases in the elderly. Rationale and design have been described previously [21]. The Rotterdam Study has been approved by the Medical Ethics Review Board of Erasmus Medical Center 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.

We studied participants of three cohorts from the Rotterdam Study. From the first cohort we had measurements at two time points, at baseline (started 1989), named RS-I-1 and at the second follow up (started 1997), named RS-I-3 (For timeline and subjects per cohort included see Figure 1). The baseline measurements of the second and third cohort are named RS-II-1 and RS-III-1, respectively (Figure 1). RS-III-1 constituted younger subjects from 45 years onwards whereas the other two cohorts constituted subjects aged 55 years and older.

In all cohorts, serum calcium, phosphate, 25-hydroxyvitamin D (25(OH) D), estradiol and testosterone levels were measured. Except for RS-I-1, blood was drawn in a fasting state. Additionally, only in RS-I-1, albumin, alkaline phosphatase (ALP) and $1,25(OH)_2D_3$ levels were measured. In all cohorts, women were interviewed for having previously used hormone replacement therapy (HRT), no data on current use of HRT was documented. In RS-II-1 and RS-III-1, women were interviewed for their current hormonal status as being pre-/perimenopausal or postmenopausal. Perimenopausal state is defined as women within one year of their last menstruation.

Assay Methods

Serum samples from subjects were analyzed at the Department of Internal Medicine of the Erasmus MC Rotterdam, The Netherlands.

Serum albumin, total serum calcium, inorganic phosphate and ALP were measured using the Hitachi 917 Analyzer (Roche, Mannheim, Germany). The calcium-phosphate product was calculated by multiplying the subjects' total serum calcium and phosphate levels and expressed in $mmol^2/L^2$. Since Payne in 1973 reported serum albumin levels to be of influence, we calculated corrected serum calcium levels using the formula: Calcium (corrected) = calcium measured + 0.02 (42 - albumin measured) in RS-I-1 [22].

For the quantitative determination of serum $1,25(OH)_2D_3$ and 25(OH)D before 1997, concerning only RS-I-1, ^{125}I-radioimmunoassays (RIA) were used (from IDS, Boldon, UK and DiaSorin, Stillwater, MN, USA; respectively). After 1997, serum 25(OH)D concentration were measured using electrochemiluminescence immunoassays (COBAS < Roche Diagnostics GmbH, Germany) this concerned RS-I-3, RS-II-1 and RS-III-1.

Serum estradiol and testosterone levels were determined by coat-a-count RIA (Siemens Diagnostics, Webster, TX). Due to limited amount of plasma per subject, not all hormone levels could be determined in all subjects.

Covariates

Several covariates that are known to differ between the sexes and covariates that potentially influence the outcome variables serum calcium and phosphate levels were included in the various statistical models used. In all cohorts, the covariates that were included in the analyses are age, body mass index (BMI), smoking status, 25(OH)D, estimated glomerular filtration rate (eGFR). Additionally, in RS-I-1, serum measurements of albumin, ALP and $1,25(OH)_2D_3$ (that were only

available in RS-I-1) were included. The covariates testosterone and estradiol were addressed in separate analyses to evaluate the magnitude of these sex specific hormones on the outcome variables.

Body mass index (BMI) was calculated as the body mass divided by the square of the body height, measured in standing position without shoes, and expressed in kg/m^2.

The Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine levels and the Modification of Diet in Renal Disease (MDRD) study equation were applied to estimate eGFR (mL/min) [23,24]. All other covariates were directly measured, as described in the paragraph on assay methods.

Statistical Analyses

Data from RS-I-3, RS-II-1 and RS-III-1 were pooled (and from now on called "the pooled dataset") in order to evaluate overall sex differences for serum calcium and phosphate. RS-I-I was analyzed separately because of non-fasting condition and availability of additional covariates. Sex differences between serum calcium and phosphate were tested using analysis of covariance (ANCOVA) in a general linear model with either serum calcium or phosphate as dependent variable and sex as a fixed factor, adjusted for age, BMI, smoking, and estimated Glomerular Filtration Rate (eGFR). Pooled dataset analyses were additionally adjusted for cohort with RS-I-3 being given dummy variables coded 0-0-1, RS-II-1 given dummy variables coded 0-1-0 and RS-III-1 given dummy variable 1-0-0 to correct for unexpected categorical effects. In RS-I-1, additional adjustments for serum albumin levels were carried out. Effect size was calculated for the outcome variables according to Cohen's D with dividing the difference in the mean between the pooled standard deviation [25].

To evaluate age in relation with serum calcium and phosphate, subjects in the pooled dataset were stratified into consecutive decades and stratified by sex. Age-

group differences were tested per sex using ANCOVA in a general linear model and adjusted for BMI, smoking, eGFR and cohort.

In order to evaluate the influence of 25(OH)D, $1,25(OH)_2D_3$ and sex hormones on sex differences in serum calcium and phosphate levels, we tested the change in the beta-coefficient of observed sex differences in a linear regression model in RS-I-1 and in the pooled dataset separately. A change of > 20% in the beta-coefficient, after correction for the specific variable, indicated the variable to have an important influence on the observed sex differences. Sex stratified two-tailed partial correlation analyses in the pooled dataset were performed to test for correlations between the sex-hormones estradiol and testosterone and the electrolytes serum calcium and phosphate. Analyses were controlled for age, BMI, eGFR, smoking and cohort.

Furthermore, we stratified women according to their hormonal state into three groups: pre-/perimenopausal women, postmenopausal women that previously used HRT, and postmenopausal women that never used HRT. We assessed differences in serum calcium and phosphate levels between these three groups using ANCOVA in a general linear model.

For the pooled dataset, only subjects with availability of all serum parameters were included in the analyses. For RS-I-1 due to limited amount of subjects with determination of the covariates 25(OH)D, $1,25(OH)_2D_3$ serum estradiol and testosterone measurements, with only 138 men and 162 women having all covariate measurements available, two subsets were created. The first included subjects with additional measurements of 25(OH)D and $1,25(OH)_2D_3$ (n=1046), and a second subset of subjects with estradiol and testosterone measurements (n=588).

Statistical analyses were performed using SPSS (version 23). Due to the explorative nature of the study, statistical significance was defined as $p < 0.05$.

Results

Sex differences

Patient characteristics are described in Table 1. In RS-I-1 (n = 2688 subjects) and in the pooled dataset (n = 9258 subjects), women had higher serum calcium and phosphate levels compared to men ($p = 2.5x10^{-5}$ and $p = 5.4x10^{-41}$ for serum calcium and $p = 8.1x10^{-86}$ and $p = 0$ for serum phosphate, in RS-I-I and in the pooled dataset, respectively; Table 2). The effect size for serum calcium was small, $r = 0.12$ in RS-I-1 and $r = 0.28$ in the pooled dataset, whereas the effect size for serum phosphate was high, $r = 0.77$ in RS-I-1 and $r = 1.01$ in the pooled dataset. As a consequence, the calcium-phosphate product was higher in women compared to men (Table 2). In RS-I-1 and in the pooled dataset, 25(OH)D levels were significantly lower in women compared to men (Table 2). Levels of $1,25(OH)_2D_3$, only determined in RS-I-1, were not significantly different between the sexes. ALP levels, also determined in RS-I-1 only, were higher in women compared to men ($p = 0.04$) but the effect size was small, $r = 0.09$.

Regression lines for serum calcium and phosphate levels plotted against age are depicted in Figure 2A and B. Interaction analysis of age and sex showed an interaction for age and sex on serum calcium levels with a standardized beta coefficient of 0.43 (p-value = $1.7x10^{-9}$) for the interaction term age*sex after adjustment for BMI, eGFR and smoking. Age and sex were independently associated with serum phosphate levels but the interaction term age*sex was not significant with a standardized beta coefficient of 0.07 (p-value = 0.3) after adjustment for BMI, eGFR and smoking. After stratification of subjects into age decades, serum calcium and phosphate showed a relation with age in both men and women with calcium and phosphate levels being lower in each consecutive decade (Table 3).

Sensitivity analyses related to serum albumin levels

As a large proportion of serum calcium is bound to albumin, we evaluated sex differences in serum albumin levels. Serum albumin levels, which were only determined in RS-I-1, were not significantly different between men and women (Table S1). After correcting serum calcium for serum albumin levels, according to the formula described in Material and methods, the sex differences in serum calcium levels remained with women having higher corrected serum calcium levels compared to men (Table S1).

To exclude an impact of high or low albumin levels on calcium levels, we performed sensitivity analyses, restricting our data to only those subjects with albumin levels within a narrow range (38-42 g/L). In this subset, accounting for 42% of the total RS-I-1 dataset, we found similar results as in the overall data (Table S1).

Effects of potential confounders of sex hormones, HRT, vitamin D and ALP on the sex differences in serum calcium and phosphate

To further investigate sex differences in serum calcium and phosphate homeostasis, we considered the role of several additional covariates including 25(OH)D, sex hormones and previous HRT in the pooled dataset, and additionally of $1,25(OH)_2D_3$ and ALP in RS-I-1.

Vitamin D does not influence sex differences in serum calcium and phosphate levels

In linear regression models, there was no change in effect size in the association between sex and serum calcium or phosphate levels after the adjustment for 25(OH)D. Adjustments for ALP or $1,25(OH)_2D_3$ levels in RS-I-1 did not change the effect size of sex as well (Table S2).

The influence of estradiol and testosterone levels on serum calcium and phosphate levels

The influence of the sex hormones estradiol and testosterone on sex differences in serum calcium and phosphate levels was assessed by calculating the change in beta coefficient using linear regression models (Table 4). The effects of adjustment for serum estradiol on serum calcium were inconsistent. Serum estradiol adjustments enhanced the effect size of the association between sex and serum calcium by 22.9% in RS-I-1, but it diminished the effect size by 21.4% in the pooled dataset. Serum testosterone adjustments did not change the association between sex and serum calcium in RS-I-1, (change < 20%), but it did enhance the effect size by 32.1% for sex differences in the pooled dataset (Table 4). The effect size for the association between sex and serum phosphate levels was diminished in both RS-I-1 (27.8%) and the pooled dataset (37.3%) after adjusting for serum testosterone levels, but sex differences remained statistically significant for serum phosphate while no change in effect size for sex differences was seen after adjusting for serum estradiol levels.

After stratification by sex, a negative correlation was seen between estradiol and serum calcium in both sexes but not between testosterone and serum calcium. Both estradiol and testosterone were negatively correlated with serum phosphate in both sexes (Table 5).

HRT use influences serum phosphate levels but not serum calcium levels

To address the influence of the hormonal status and the use of HRT on total serum calcium, phosphate and 25(OH)D levels, we stratified women into three groups: pre-/perimenopausal women, postmenopausal women that previously used HRT, and postmenopausal women that never used HRT (Table 6). Previous users had significantly higher 25(OH)D levels compared to non-users in RS-I-1 and in the pooled dataset. Pooled dataset analyses showed significant differences for phosphate between all groups, with postmenopausal women who never used

HRT having the highest serum phosphate levels followed by previous users of HRT, pre-/perimenopausal women, and men having lowest serum phosphate levels. Serum calcium levels were significantly higher in postmenopausal women compared to men and pre-/perimenopausal women, only after adjusting for the variables age, BMI, eGFR and smoking. There was no significant difference between postmenopausal women and previous users of HRT.

Discussion

The pooled analysis in three cohorts from the Rotterdam Study has revealed sexual dimorphism for serum calcium and phosphate levels in subjects aged 45 years and older. Serum calcium, phosphate and the calcium-phosphate product were significantly higher in women compared to men. Both serum calcium levels, in a sex-dependent manner, and serum phosphate levels, in a sex-independent manner, declined with aging. There are a few older and usually small-sized studies that have found higher serum calcium levels in elderly women compared to men, although not always consistently [9,11,12], while several studies have shown that postmenopausal women have higher serum phosphate levels than men of similar age [9,13-17,26]. The findings from the current study have now confirmed sexual dimorphism both in serum calcium and phosphate at the population level.

Sex differences in serum calcium and phosphate are not influenced by bone turnover or 25(OH)D

Since calcium and phosphate are mainly stored in bone, we assessed sex differences in calcium and phosphate in relation to bone turnover as reflected by serum ALP. However, adjustment for ALP levels, which were higher in women compared to men, as is known in the postmenopausal state due to estrogen deficiency [27], did not influence sex differences in calcium and phosphate levels in our study. This suggests that a higher bone turnover in postmenopausal women compared to men does not fully explain the observed increases in serum calcium and phosphate levels. Also, adjustment for 25(OH)D levels, which were lower in women, did not

influence serum calcium and phosphate levels.

Sex differences in serum calcium are not explained by differences in serum testosterone and estradiol, but serum testosterone influences sex differences in serum phosphate

Sex hormone actions are a potential cause of the observed sex differences in serum calcium and phosphate levels. The effects of serum estradiol and testosterone levels on sex differences in serum calcium are inconsistent between RS-I-1 and the pooled dataset of RS-I-3, RS-II-1 and RS-III-1. Sex differences in serum phosphate levels were not modified by serum estradiol levels and decreased after adjusting for serum testosterone levels. This suggests that differences in sex hormones, especially serum testosterone, may explain in part the sex differences in serum phosphate after the age of 45. We cannot conclude with certainty that serum testosterone but not serum estradiol is important in the age-related sex differences of phosphate regulation since testosterone can be converted into estradiol through the activity of the enzyme aromatase cytochrome P-450 [28]; therefore, the action at the tissue level might be driven by estradiol as well. A study of 59 elderly men with suppressed endogenous production of estradiol and testosterone through administration of a long-acting GnRH agonist demonstrated that estrogen supplementation has been able to prevent an increase in bone resorption markers without changing serum calcium and phosphate levels. Depletion of estrogen but not testosterone in these men resulted in increased serum calcium levels and increased bone resorption markers, while both estradiol and testosterone suppression independently increased serum phosphate levels [29]. Estradiol is able to induce phosphaturia in a PTH-independent pathway and might be a potent stimulus for FGF23 secretion [30]. Hence, the drop in estradiol associated with menopause could lead to diminished phosphate excretion by the kidneys, which may explain the higher serum phosphate levels after menopause as seen in our study as well as documented by others [9,13-17]. Therefore, both estradiol and testosterone may play a role in the regulation of serum calcium and phosphate but to what extent and whether there are sex differences in the

regulatory pathways is still unclear.

Calcium homeostasis is predominantly regulated at the level of the parathyroid glands, the kidneys, the intestines and bone. Both human and animal studies have found that estradiol reduces renal calcium excretion and increases intestinal calcium absorption suggesting sex differences in renal and intestinal calcium handling [31,32]. However, this is not in line with our findings of higher levels of serum calcium in postmenopausal women when estradiol levels drop, suggesting an additional regulatory mechanism. Nordin *et al*. postulated that the sexual dimorphism in calcium handling could be due to a change in PTH and increased sensitivity for the PTH action on bone after menopause [8,33]. Unfortunately, no serum PTH levels were available in our cohort. Hypothetically, though, higher serum calcium levels would be expected to lead to decreased PTH levels and decreased conversion of 25(OH)D into $1,25(OH)_2D_3$. We have observed 25(OH) D, but not $1,25(OH)_2D_3$, to be significantly lower in women compared to men but adjustments for these metabolites did not influence sex differences in serum calcium and phosphate.

A role for estrogen in calcium homeostasis is also suggested by the observation that HRT can decrease serum calcium levels, as previously described by Prince *et al*. [34]. In our study, we did not find significant differences in serum calcium or phosphate levels between postmenopausal women that previously used HRT and those that never used HRT; but since we did not have information on how many previous users of HRT in the past were still using it, or the time elapsed since HRT beginning, a potential association could be obscured.

Our study showed that sex differences in serum phosphate were partly driven by serum testosterone levels, but not by serum estradiol levels. In a study in community-dwelling older men, higher serum estradiol and testosterone levels have been independently associated with lower serum phosphate levels, also after adjustment for FGF23 levels, one of the major phosphate regulating hormones [35,36]. Meng *et al*. could not attribute the inverse association between the sex

hormones and phosphate to increased bone turnover since adjustments for bone mineral density and ALP did not influence the association [35]. Most studies directly assessing the influence of sex steroids on serum phosphate and calcium levels focused on serum estradiol, although an increasing body of evidence suggests a relationship between serum phosphate and testosterone [35,37]. As Meng *et al*. mentioned, there have not been many studies on the influence of testosterone on phosphate handling [35]. Further research to elucidate whether testosterone has potential direct effects on phosphate handling in the kidneys and intestines and on its regulatory hormones (PTH, FGF23 and klotho) will lead to a greater understanding of how testosterone influences the bone-kidney axis.

Relevance of findings

Serum calcium and phosphate levels are correlated with cardiometabolic diseases and mortality, and understanding the underlying mechanisms is a key priority. Our study has added comprehensive data to previous, smaller-scale studies demonstrating sexual dimorphism in serum calcium and phosphate levels.

Lorenzo *et al*. have found serum calcium and the calcium-phosphate product but not phosphate to be associated with the incidence of type 2 diabetes in both sexes [38]. Moreover, Larsson *et al*. showed that a genetic predisposition to higher serum calcium levels was associated with increased myocardial infarction and coronary artery disease [39].

Dhingra *et al*. showed that serum phosphate levels, but not serum calcium levels, have been associated with the composite endpoint of fatal and non-fatal cardiovascular disease (CVD) events in non-chronic kidney disease (CKD) subjects [15] and a recent meta-analysis by Bai *et al*. in 120.269 subjects showed serum phosphate to be associated with all-cause mortality in men but not in women [40].

Foley *et al*. (2009) have found in a large prospective study that higher serum

phosphate levels in young adults are associated with higher scores of coronary artery calcium levels [41].

The higher serum levels of calcium and phosphate with the resulting higher calcium-phosphate product that we found in postmenopausal women compared to men of the same age may thus be clinically relevant. It may even be speculated that higher levels of serum calcium and phosphate after menopause may partly underlie the rise in CVD that is observed in women after menopause [42,43].

Strengths and limitations

The strength of this study is the availability of data in a large number of men and women in the Rotterdam Study, with prospective information in the first cohort and a large number of covariates, including serum estradiol, testosterone, vitamin D, ALP levels and previous HRT use, that have allowed us to study the influence of these variables on calcium and phosphate homeostasis. Despite the relatively small sex differences in serum calcium and phosphate levels, our findings may be important to explain underlying pathways for these sex differences and may be relevant in light of the increasing number of studies showing that serum levels of calcium and phosphate in the general population are related to multiple diseases and mortality [6,16,18,44]. Correcting for albumin in various ways has not influenced our findings of sex-based differences in total serum calcium levels in RS-I-1.

Limitations of our study are the lack of serum PTH and FGF23 levels and urinary excretion of calcium and phosphate. Furthermore, the fact that serum phosphate in RS-I-1 in our study was determined in a non-fasting state may have potentially influenced some of our findings [45]. Moreover, data on the duration of HRT and current use was not documented, which could have obscured a possible effect of HRT on serum calcium and phosphate levels. Lastly, the results are not generalizable to non-Caucasian populations and despite adjustment for multiple covariates, we cannot exclude the possibility of residual confounding.

Conclusions

This study demonstrates sexual dimorphism in serum calcium and phosphate levels with postmenopausal women having significantly higher levels compared to men of similar age. The sex differences in serum phosphate but not calcium are consistently decreased by adjustment for serum testosterone but not for serum estradiol, vitamin D or ALP. Based on the relations between serum calcium and phosphate levels and morbidity, especially cardiometabolic diseases and mortality, studies providing insight into the mechanisms behind the origin of these sex differences may be of great relevance for public health and sex-based medicine.

Table 1: General characteristics in men and women in the RS depicted as mean with 95% CI (Confidence Intervals) between brackets.

	RS-I-1		ANCOVA	Pooled dataset RS-I-3, RS-II-1 and RS-III-1[4]		ANCOVA
	Men	Women	p value*	Men	Women	p value*
No.	1009	1679		4043	5215	
Age (years)	69.6 (69.2-70.1)	71.2 (70.8-71.7)	$2.0x10^{-6}$	64.5 (64.2-64.8)	65.3 (64.9-65.5)	0.001
BMI (kg/m^2)[1]	25.7 (25.5-25.9)	26.6 (26.4-26.8)	$3.1x10^{-10}$	27.0 (26.9-27.1)	27.5 (27.3-27.6)	$2.2x10^{-7}$
eGFR (mL/min)[2]	79.8 (78.8-80.8)	69.4 (68.7-70.1)	$7.3x10^{-17}$	80.0 (79.5-80.5)	78.7 (28.3-79.1)	0.10
Estradiol (pmol/L)[3]	46.73 (43.73-49.73)	14.48 (12.85-16.12)	$1.6x10^{-64}$	104.21 (102.95-105.46)	61.47 (58.69-64.25)	$6.9x10^{-134}$
Testosterone (nmol/L)[3]	10.60 (10.17-11.02)	1.24 (1.16-1.31)	$3.8x10^{-198}$	17.17 (16.99-17.35)	0.94 (0.92-0.96)	0

BMI = body mass index; eGFR = estimated glomerular filtration rate according to Modification of Diet in Renal Disease (MDRD) study equation

* P-value according to Welch test due to inhomogenity of variances between both groups

[1] Adjusted for age

[2] Adjusted for age, smoking, BMI

[3] Adjusted for age, smoking, BMI and eGFR

[4] The pooled dataset was additionally adjusted for cohort

Table 2: Serum calcium and phosphate parameters depicted as mean with 95% CI between brackets.

	RS-I-1		Effect size	ANCOVA	Pooled dataset RS-I-3, RS-II-1 and RS-III-1[2]		Effect size	ANCOVA
	Men	Women	r	p value	Men	Women	r	p value
No.	1009	1679			4043	5215		
Serum calcium (mmol/L)[1]	2.356 (2.347-2.364)	2.372 (2.366-2.379)	0.12	$2.5x10^{-5}$	2.415 (2.412-2.418)	2.443 (2.440-2.446)	0.28	$5.4x10^{-41}$
Serum phosphate (mmol/L)[1]	1.090 (1.079-1.102)	1.230 (1.221-1.238)	0.77	$8.1x10^{-86}$	1.024 (1.019-1.028)	1.171 (1.167-1.175)	1.01	0
Calcium-phosphate product ($mmol^2/L^2$)[1]	2.574 (2.543-2.606)	2.920 (2.898-2.943)	0.71	$5.9x10^{-77}$	2.475 (2.462-2.487)	2.864 (2.853-2.874)	0.99	0
Alkaline Phosphatase IU/L[1]	80.42 (78.68-82.16)	82.92 (81.64-84.20)	0.09	0.04	ND	ND		
25(OH)D (nmol/L)[1]	69.13 (66.53-71.73)	59.82 (57.81-61.83)	0.35	$2.3x10^{-12}$	59.90 (59.03-60.76)	53.55 (52.81-54.28)	0.23	$8.1x10^{-23}$
$1,25(OH)_2D_3$ (pmol/L)[1]	109.07 (106.22-111.92)	105.43 (103.18-107.68)	0.12	0.65	ND	ND		

25(OH)D = 25-hydroxyvitamin D; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D; ND = not determined.

[1] Adjusted for age, smoking, BMI and eGFR

[2] The pooled dataset is additionally adjusted for cohort

Table 3: Serum calcium and phosphate levels across consecutive decades stratified by sex in the pooled dataset of RS-I-3, RS-II-1 and RS-III-1.

	Men						Women					
Decades(yrs)	45-54	55-64	65-74	75-84	>85*		45-54	55-64	65-74	75-84	>85*	
No.	590	1708	1126	545	73	p value	701	2169	1351	842	151	p value
Calcium (mmol/L)	2.454 (2.445-2.462)	2.419 (2.414-2.424)	2.401 (2.395-2.406)	2.392 (2.384-2.401)	2.394 (2.375-2.413)	$1.1x10^{-6}$	2.453 (2.445-2.461)	2.447 (2.442-2.451)	2.435 (2.430-2.440)	2.438 (2.432-2.445)	2.437 (2.416-2.458)	$5.0x10^{-6}$
Phosphate (mmol/L)	1.060 (1.046-1.073)	1.019 (1.012-1.026)	1.017 (1.008-1.025)	1.012 (1.001-1.023)	1.038 (1.008-1.068)	0.002	1.175 (1.163-1.187)	1.179 (1.173-1.185)	1.170 (1.163-1.178)	1.157 (1.148-1.167)	1.136 (1.116-1.157)	$2.1x10^{-5}$

Analyses were adjusted for BMI, eGFR, smoking and cohort. Values are depicted as mean with 95% CI

* Due to limited numbers of participants above 85 years this group ranges 85-96.7 years in men and 85-101 years in women

Table 4: Influence of serum testosterone and estradiol on sex differences in serum calcium and phosphate levels in RS-I-1 and the pooled dataset of RS-I-3, RS-II-1 and RS-III-1

		Serum calcium (mmol/L)		Serum phosphate (mmol/L)	
		Unstandardized Beta-coefficient sex (95% CI)	% change beta-coefficient from model 1	Unstandardized Beta-coefficient sex (95% CI)	% change beta-coefficient from model 1
RS-I-1	Model 1	0.035 (0.013-0.057)*		0.162 (0.132-0.192)*	
N=588	Model 2	0.043 (0.014-0.071)*	22.9%	0.147 (0.109-0.186)*	-9.3%
	Model 3	0.031 (-0.017-0.079)	-11.4%	0.117 (0.052-0.182)*	-27.8%
	Model 4	0.033 (-0.015-0.082)	-5.7%	0.114 (0.048-0.179)*	-29.4%
Pooled dataset RS-I-3, RS-II-1 and RS-III-1‡	Model 1	0.028 (0.024-0.033)*		0.150(0.144-0.156)*	
N=9270	Model 2	0.022 (0.018-0.026)*	-21.4%	0.140 (0.134-0.146)*	-6.7%
	Model 3	0.037 (0.028-0.046)*	32.1%	0.094 (0.081-0.108)*	-37.3%
	Model 4	0.036 (0.027-0.045)*	28.6%	0.092 (0.079-0.105)*	-38.7%

Model 1: adjusted for age, BMI, eGFR and smoking

Model 2: model 1 and estradiol

Model 3: model 1 and testosterone

Model 4: model 1 and estradiol and testosterone

* Significant sex difference in the model

‡ Pooled dataset models additionally adjusted for cohort

Table 5: Partial correlation analysis in the pooled data set of RS-I-3, RS-II-1 and RS-III-1 for serum calcium and phosphate with estradiol and testosterone stratified by sex

		Serum calcium		**Serum phosphate**	
		r	p value	r	p value
Men	Estradiol	-0.173	2.0×10^{-28}	-0.151	5.9×10^{-22}
	Testosterone	0.044	0.005	-0.110	8.4×10^{-12}
Women	Estradiol	-0.158	7.2×10^{-30}	-0.184	2.7×10^{-40}
	Testosterone	0.011	0.43	-0.040	0.004

Analyses are adjusted for age, BMI, eGFR, smoking and cohort

Table 6: Parameters of calcium and phosphate homeostasis with 95% Confidence Intervals (CI) with women stratified into one of three groups: a pre- and perimenopausal group, a postmenopausal women group that previoustly used HRT, and a group of postmenopausal women that never used HRT.

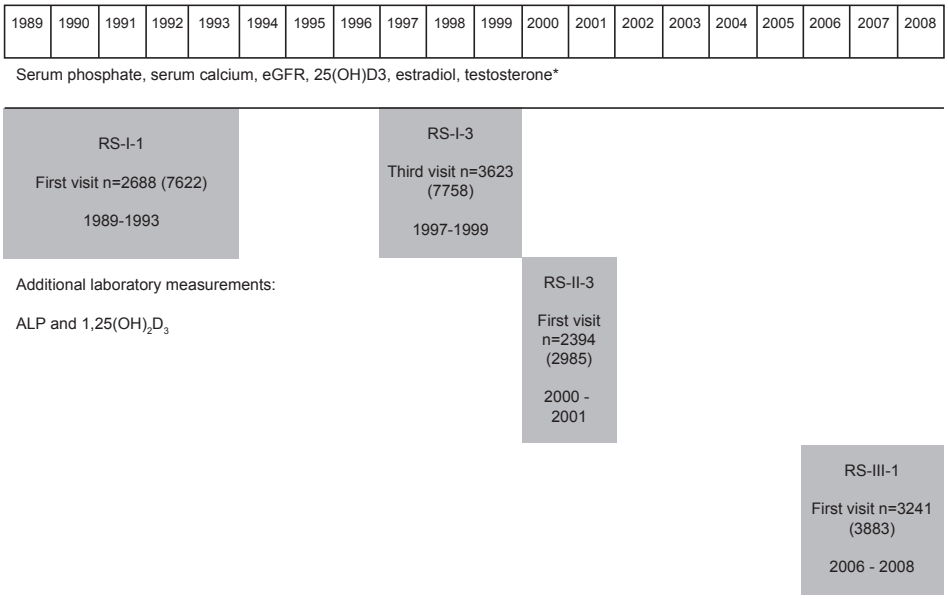
	Men (95% CI)	Pre- and perimenopausal women (95% CI)	Ever-user HRT (95% CI)	Postmenopausal women (95% CI)	AN(C)OVA p value[l]
RS-I-1					
Number	1009	NA	252	1377	
Serum calcium (mmol/L)	2.356 (2.347-2.364)	NA	2.372 (2.356-2.388)	2.372 (2.365-2.379)	0.59
Serum phosphate (mmol/L)	1.090 (1.079-1.102)	NA	1.228 (1.204-1.252)	1.231 (1.222-1.240)	0.39
25(OH)D (nmol/L)	69.13 (66.53-71.73)	NA	65.75 (60.24-71.26)	58.24 (56.14-60.33)	0.01
Estradiol (pmol/L)	46.727 (43.727-49.727)	NA	14.759 (9.981-19.537)	13.990 (12.289-15.692)	0.88
Testosterone (nmol/L)	10.595 (10.168-11.022)	NA	1.162 (0.973-1.351)	1.232 (1.150-1.313)	0.41
Pooled dataset RS-I-3, RS-II-1 and RS-III-1[‡]					
Number	4043	413	847	3383	
Serum calcium (mmol/L)	2.415 (2.412-2.418)	2.444 (2.432-2.445)	2.443 (2.436-2.450)	2.444 (2.441-2.447)	$5.3x10^{-5}$
Serum phosphate (mmol/L)	1.024 (1.019-1.028)	1.144 (1.129-1.159)	1.165 (1.154-1.175)	1.175 (1.170-1.179)	$6.0x10^{-17}$
25(OH)D (nmol/L)	59.90 (59.03-60.76)	61.47 (58.67-64.26)	59.07 (57.19-60.95)	53.11 (52.21-54.01)	$5.0x10^{-6}$
Estradiol (pmol/L)	104.21 (102.95-105.46)	221.67 (196.69-246.65)	61.84 (56.15-67.54)	45.89 (44.15-47.63)	$2.3x10^{-187}$
Testosterone (nmol/L)	17.17 (16.99-17.35)	0.85 (0.81-0.88)	0.89 (0.84-0.93)	0.95 (0.92-0.98)	0.39

All parameters are adjusted for age, smoking, BMI and eGFR

[l] p-value of the AN(C)OVA concerns analyses across women

[‡] Pooled dataset additionally adjusted for cohort

Figure 1: timeline Rotterdam study



n= number of subjects included from number of total subjects between brackets

Figure 2A

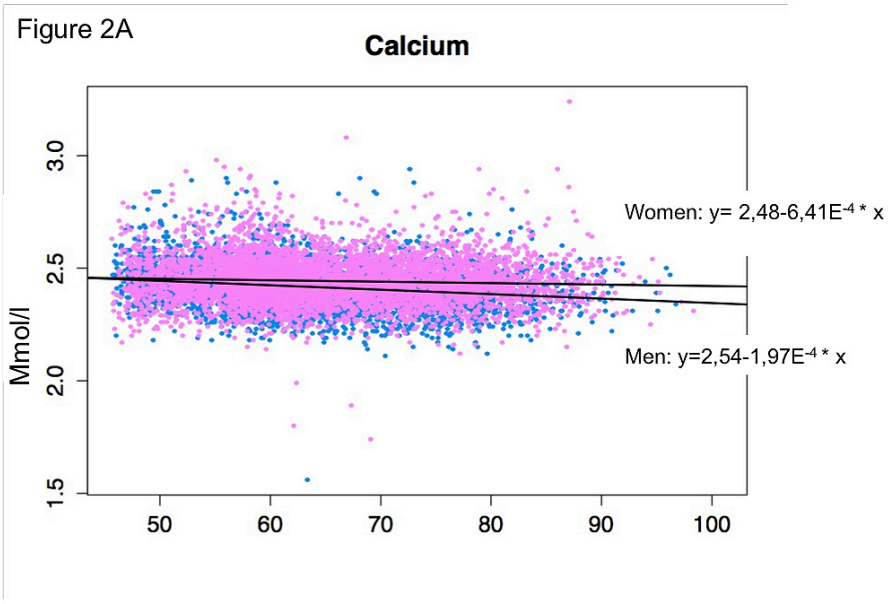


Figure 2B

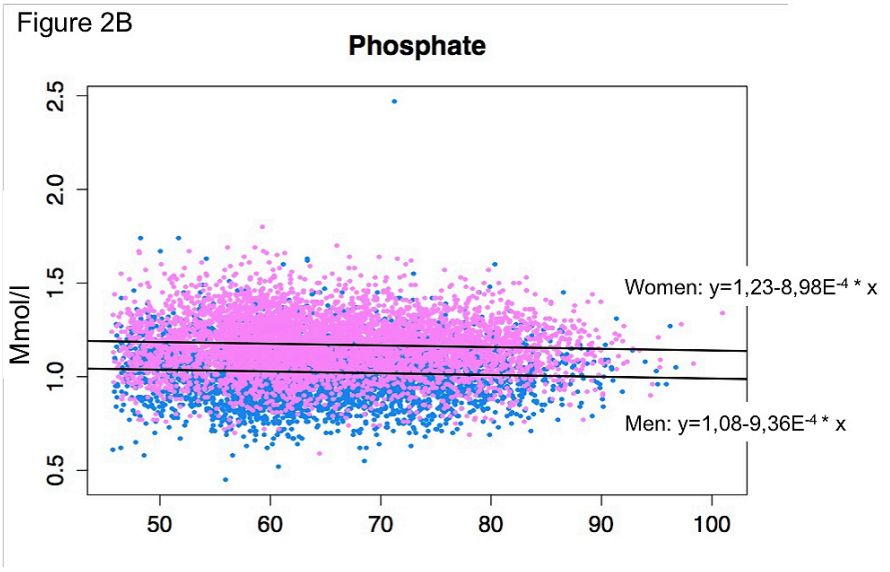


Figure 2A en 2B:

2A: Scatterplots of serum calcium plotted against age with men in blue and women in pink.

2B: Scatterplots of serum phosphate plotted against age with men in blue and women in pink.

References

1. **Peacock M**. Calcium metabolism in health and disease. *Clin J Am Soc Nephrol* (5 Suppl) 1:S23-30. doi:10.2215/CJN.05910809

2. **Renkema KY, Alexander RT, Bindels RJ, Hoenderop JG**. Calcium and phosphate homeostasis: concerted interplay of new regulators. *Ann Med* 2008 40 (2):82-91. doi:784377218 [pii]
10.1080/07853890701689645

3. **Kolek OI, Hines ER, Jones MD, LeSueur LK, Lipko MA, Kiela PR, Collins JF, Haussler MR, Ghishan FK**. 1alpha,25-Dihydroxyvitamin D3 upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport. *Am J Physiol Gastrointest Liver Physiol* 2005; 289 (6):G1036-42. doi:00243.2005 [pii]10.1152/ajpgi.00243.2005

4. **Bansal VK. Serum Inorganic Phosphorus**. 1990. doi: NBK310 [book accession]

5. **Jorde R, Sundsfjord J, Fitzgerald P, Bonaa KH**. Serum calcium and cardiovascular risk factors and diseases: the Tromso study. *Hypertension* 1999; 34 (3):484-90.

6. **Haglin L, Backman L, Tornkvist B.** A structural equation model for assessment of links between changes in serum triglycerides, -urate, and -glucose and changes in serum calcium, -magnesium and -phosphate in type 2 diabetes and non-diabetes metabolism. *Cardiovasc Diabetol* 2011; 10:116. doi:1475-2840-10-116 [pii]10.1186/1475-2840-10-116

7. **Riggs BL, Khosla S, Melton LJ, 3rd**. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J Bone Miner Res* 1998; 13 (5):763-773. doi:10.1359/jbmr.1998.13.5.763

8. **Nordin BE, JM WI, Clifton PM, McArthur R, Scopacasa F, Need AG, Morris HA, O'Loughlin PD, Horowitz M**. A longitudinal study of bone-related biochemical changes at the menopause. *Clin Endocrinol (Oxf)* 2004; 61(1):123-130. doi:10.1111/j.1365-2265.2004.02066.xCEN2066 [pii]

9. **Keating FR, Jr., Jones JD, Elveback LR, Randall RV**. The relation of age and sex to distribution of values in healthy adults of serum calcium, inorganic phosphorus, magnesium, alkaline phosphatase, total proteins, albumin, and blood urea. *J Lab Clin Med* 1969; 73(5):825-834. doi:0022-2143(69)90096-1 [pii]

10. **Lindgarde F**. Potentiometric determination of serum ionized calcium in a normal human population. *Clin Chim Acta* 1972; 40(2):477-484. doi:0009-8981(72)90360-9 [pii]

11. **Nordin BE, Need AG, Morris HA, Horowitz M**. Biochemical variables in pre- and postmenopausal women: reconciling the calcium and estrogen hypotheses. *Osteoporos Int* 1999; 9 (4):351-357. doi:709 [pii]

12. **Roof BS, Piel CF, Hansen J, Fudenberg HH**. Serum parathyroid hormone levels and serum calcium levels from birth to senescence. *Mech Ageing Dev* 1976; 5(4):289-304.

13. **Cirillo M, Ciacci C, De Santo NG**. Age, renal tubular phosphate reabsorption, and serum phosphate levels in adults. *N Engl J Med* 2008; 359(8):864-6. doi:10.1056/NEJMc0800696359/8/864 [pii]

14. **de Boer IH, Rue TC, Kestenbaum B**. Serum phosphorus concentrations in the third National Health and Nutrition Examination Survey (NHANES III). *Am J Kidney Dis* 2009; 53(3):399-407. doi:10.1053/j.ajkd.2008.07.03 6S0272-6386(08)01232-8 [pii]

15. **Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB, Sr**., Gaziano JM, Vasan RS. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007; 167(9):879-85. doi:167/9/879 [pii]10.1001/archinte.167.9.879

16. **Onufrak SJ, Bellasi A, Cardarelli F, Vaccarino V, Muntner P, Shaw LJ, Raggi P**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009; 169 (1):67-77. doi:10.1093/aje/kwn285kwn285 [pii]

17. **Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G**. Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005; 112 (17):2627-33. doi:112/17/2627 [pii]10.1161/CIRCULATIONAHA.105.553198

18. **Barbagallo M, Dominguez LJ, Licata G, Resnick LM**. Effects of aging on serum ionized and cytosolic free calcium: relation to hypertension and diabetes. *Hypertension* 1999; 34 (4 Pt 2):902-6.

19. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK.** Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998; 31(4):607-17. doi:S0272638698000912 [pii]

20. **Appelman Y, van Rijn BB, Ten Haaf ME, Boersma E, Peters SA**. Sex differences in cardiovascular risk factors and disease prevention. *Atherosclerosis* 2015. doi:S0021-9150(15)00061-1 [pii]10.1016/j.atherosclerosis.2015.01.027

21. **Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, Klaver CC, Nijsten TE, Peeters RP, Stricker BH, Tiemeier HW, Uitterlinden AG, Vernooij MW**. The Rotterdam Study: 2016 objectives and design update. *Eur J Epidemiol* 2015; 30 (8):661-708. doi:10.1007/s10654-015-0082-x

22. **Payne RB, Little AJ, Williams RB, Milner JR**. Interpretation of serum calcium in patients with abnormal serum proteins. *Br Med J* 1973; 4(5893):643-6.

23. **Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D**. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; 130(6):461-70.

24. **Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, Ckd EPI**. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009; 150(9):604-12

25. **Colman AM**. A Dictionary of Psychology. 2008. 3 edn. Oxford University Press.

26. **Boyd JL, Delost ME, Holcomb JP**. Calcium, phosphorus, and alkaline phosphatase values of elderly subjects. *Clin Lab Sci* 1998; 11 (4):223-7.

27. **Ebeling PR, Atley LM, Guthrie JR, Burger HG, Dennerstein L, Hopper JL, Wark JD.** Bone turnover markers and bone density across the menopausal transition. *J Clin Endocrinol Metab* 1996; 81 (9):3366-3371. doi:10.1210/

jcem.81.9.8784098

28. **Meinhardt U, Mullis PE**. The aromatase cytochrome P-450 and its clinical impact. *Horm Res* 2002; 57 (5-6):145-152. doi:58374 [pii]58374

29. **Falahati-Nini A, Riggs BL, Atkinson EJ, O'Fallon WM, Eastell R, Khosla S**. Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. *J Clin Invest* 2000; 106(12):1553-1560. doi:10.1172/JCI10942

30. **Faroqui S, Levi M, Soleimani M, Amlal H.** Estrogen downregulates the proximal tubule type IIa sodium phosphate cotransporter causing phosphate wasting and hypophosphatemia. *Kidney Int* 2008; 73(10):1141-50. doi:10.1038/ki.2008.33ki200833 [pii]

31. **Arjmandi BH, Hollis BW, Kalu DN**. In vivo effect of 17 beta-estradiol on intestinal calcium absorption in rats. *Bone Miner* 1994; 26 (2):181-9.

32. **Colin EM, van Den Bemd GJ, van Aken M, Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, van Leeuwen JP.** Evidence for involvement of 17beta-estradiol in intestinal calcium absorption independent of 1,25-dihydroxyvitamin D3 level in the Rat. *J Bone Miner Res* 1999; 14(1):57-64. doi:10.1359/jbmr.1999.14.1.57

33. **Nordin BE, Need AG, Morris HA, Horowitz M, Robertson WG**. Evidence for a renal calcium leak in postmenopausal women. *J Clin Endocrinol Metab* 1991; 72(2):401-7.

34. **Prince RL**. Counterpoint: estrogen effects on calcitropic hormones and calcium homeostasis. *Endocr Rev* 1994; 15(3):301-9.

35. **Meng J, Ohlsson C, Laughlin GA, Chonchol M, Wassel CL, Ljunggren O, Karlsson MK, Mellstrom D, Orwoll ES, Barrett-Connor E, Ix JH**. Associations of estradiol and testosterone with serum phosphorus in older men: the Osteoporotic Fractures in Men study. *Kidney Int* 2010; 78(4):415-22. doi:10.1038/ki.2010.161ki2010161 [pii]

36. **Meng J, Wassel CL, Kestenbaum BR, Collins TC, Criqui MH, Lewis CE, Cummings SR, Ix JH**. Serum phosphorus levels and the spectrum of ankle-brachial index in older men: the Osteoporotic Fractures in Men (MrOS) study. *Am J Epidemiol* 2010; 171(8):909-16. doi:10.1093/aje/kwq020kwq020 [pii]

37. **Wulaningsih W, van Hemelrijck M, Michaelsson K, Kanarek N, Nelson WG, Ix JH, Platz EA, Rohrmann S**. Association of serum inorganic phosphate with sex steroid hormones and vitamin D in a nationally representative sample of men. *Andrology* 2014; 2(6):967-976. doi:10.1111/andr.285

38. **Lorenzo C, Hanley AJ, Rewers MJ, Haffner SM**. Calcium and phosphate concentrations and future development of type 2 diabetes: the Insulin Resistance Atherosclerosis Study. *Diabetologia* 2014; 57(7):1366-1374. doi:10.1007/s00125-014-3241-9

39. **Larsson SC, Burgess S, Michaelsson K**. Association of Genetic Variants Related to Serum Calcium Levels With Coronary Artery Disease and Myocardial Infarction. *JAMA* 2017; 318(4):371-80. doi:10.1001/jama.2017.8981

40. **Bai W, Li J, Liu J**. Serum phosphorus, cardiovascular and all-cause mortality in the general population: A meta-analysis. *Clin Chim Acta* 2016; 461:76-82. doi:10.1016/j.cca.2016.07.020

41. **Foley RN, Collins AJ, Herzog CA, Ishani A, Kalra PA**. Serum phosphorus levels associate with coronary atherosclerosis in young adults. *J Am Soc Nephrol* 2009; 20(2):397-404. doi:10.1681/ASN.2008020141ASN.2008020141 [pii]

42. **Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB, American Heart Association Statistics C, Stroke Statistics S**. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* 2012; 125(1):e2-e220. doi:10.1161/CIR.0b013e31823ac046

43. **de Kat AC, Dam V, Onland-Moret NC, Eijkemans MJ, Broekmans FJ, van der Schouw YT**. Unraveling the associations of age and menopause with cardiovascular risk factors in a large population-based study. *BMC Med* 2017; 15(1):2. doi:10.1186/s12916-016-0762-8

44. **Tohno Y, Tohno S, Mahakkanukrauh P, Minami T, Sinthubua A,**

Suwannahoy P, Ongkana N, Laowatthanaphong S, Azuma C. Gender difference in accumulation of calcium and phosphorus in the left coronary arteries of Thais. *Biol Trace Elem Res* 2011; 144(1-3):17-26. doi:10.1007/s12011-011-9016-y

45. **Chang AR, Grams ME**. Serum phosphorus and mortality in the Third National Health and Nutrition Examination Survey (NHANES III): effect modification by fasting. *Am J Kidney Dis* 2014; 64(4):567-573. doi:10.1053/j.ajkd.2014.04.028S0272-6386(14)00837-3 [pii]

Supplementary Material

Supplementary Table 1: Serum parameters of albumin and calcium corrected for albumin and sensitivity analyses of parameters of calcium homeostasis in a subset of men and women above 55 years in the Rotterdam Study RS-I-1 (mean with 95% CI).

Parameters	**Rotterdam Study RS-I-1**		**Effect size**	**ANOVA**
	Men	Women	r	p Value[a,b]
Albumin (g/L)[c]	42.215 (42.055-42.375)	42.076 (41.957-42.195)	0.05	0.80
Corrected calcium (mmol/L)[c]	2.352 (2.342-2.360)	2.371 (2.364-2.377)	0.14	$1.6x10^{-5}$
Subset (albumin 38-42 g/L)				
Number	401	734		
Serum calcium (mmol/L)[c]	2.342 (2.329-2.355)	2.359 (2.349-2.369)	0.12	0.029
Phosphate (mmol/L)[c]	1.073 (1.055-1.090)	1.219 (1.207-1.232)	0.85	$8.5x10^{-42}$
Calcium-phosphate product ($mmol^2/L^2$)[c]	2.515 (2.470-2.560)	2.878 (2.846-2.910)	0.81	$2.8x10^{-83}$
Alkaline phosphatase (U/L)[c]	82.170 (79.109-85.231)	84.606 (82.510-86.702)	0.08	0.39
25(OH)D (nmol/L)[c]	65.58 (61.24-69.92)	59.83 (56.56-63.11)	0.22	0.008
$1,25(OH)_2D_3$ (pmol/L)[c]	109.2 (104.20-114.25)	105.05 (101.14-109.6)	0.13	0.81

BMI = body mass index; eGFR = estimated glomerular filtration rate according to Modification of Diet in Renal Disease (MDRD) study equation; $25(OH)D_3$ = 25-hydroxyvitamin D; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D

[a] adjusted for age

[b] adjusted for age and smoking

[c] adjusted for age, BMI, smoking and eGFR

Supplementary Table 2: The influence of 25(OH)D and $1,25(OH)_2D_3$ on the sex differences in serum calcium and phosphate levels in RS-I-1 and in the pooled dataset

		Serum calcium		Serum phosphate	
		Unstandardized Beta-coefficient sex (CI)	% change beta-coefficient from model 1	Unstandardized Beta-coefficient sex (CI)	% change beta-coefficient from model 1
RS-I-1	Model 1	0.030 (0.013-0.046)		0.167 (0.143-0.191)	
N=1046	Model 2	0.027 (0.010-0.044)	-10.0%	0.162 (0.138-0.187)	-3.0%
	Model 3	0.030 (0.013-0.046)	0.0%	0.167 (0.143-0.190)	0.0%
	Model 4	0.026 (0.091-0.043)	-13.3%	0.163 (0.139-0.187)	-2.4%
Pooled dataset	Model 1	0.028 (0.024-0.032)*		0.150 (0.144-0.156)*	
N= 9258	Model 2	0.029 (0.025-0.033)*	3.6%	0.150 (0.144-0.156)*	0.0%

Model 1: adjusted for age, body mass index (BMI), estimated Glomerular Filtration Rate (eGFR) and smoking

Model 2: model 1 and 25(OH)D

Model 3: model 1 and $1,25(OH)_2D_3$

Model 4: model 1 and 25(OH)D and $1,25(OH)_2D_3$

* Significant different for sex

8

Age-dependent sex differences in calcium and phosphate homeostasis

Age-dependent sex differences in calcium and phosphate homeostasis

Authors:

W.N.H. Koek*, N. Campos-Obando*, B.C.J. van der Eerden, Y.B. de Rijke, M.A. Ikram, A.G. Uitterlinden1, J.P.T.M. van Leeuwen and M.C. Zillikens

*These authors contributed equally to this work

Status:

Manuscript ready for submission.

Abstract

Background: Sex differences in calcium and phosphate have been observed. We aimed to assess a relation with age.

Methods: We used the laboratory values of serum calcium, phosphate and albumin from three different samples (years 2005, 2010 and 2014) using the hospital information system of Erasmus MC, Rotterdam. The samples were divided into 3 age groups: 1-17, 18-44 and ≥45 years. Sex differences in calcium and phosphate were analyzed using ANCOVA, adjusting for age and serum albumin. Furthermore, sex by age interactions were determined and we analyzed differences between age groups stratified by sex.

Results: In all three samples there was a significant sex*age interaction term for serum calcium and phosphate, whose levels were significantly higher in women compared to men above 45 years. No sex differences in the younger age groups were found. In men, serum calcium and phosphate levels were highest in the youngest age group compared to age groups of 18-44 and ≥45 years. In women, serum calcium levels were also significantly higher in the age group 1-17 and the age group ≥45 years compared to the 18-44 years age group. In women, serum phosphate was different between the three different age groups with highest level in the group 1-17 years and lowest in the group 18-44 years.

Conclusion: There are age dependent sex differences in serum calcium and phosphate. Furthermore, we found differences in serum calcium and phosphate between different age groups. Underlying mechanisms for these age- and sex differences are not yet fully elucidated.

Introduction

Calcium and phosphate imbalance has been linked to several disorders such as cardiovascular disease, metabolic syndrome, osteoporosis and mortality [1-4]. Some of these conditions display marked sex-specific incidences [3, 5, 6], which might be related to sex differences in calcium and phosphate homeostasis.

In a population based study (unpublished data) we found post-menopausal women to have higher serum calcium and phosphate levels compared to men above 45 years of age. Adjustment for serum testosterone levels diminished the sex differences in serum phosphate but not serum calcium levels, while adjustment for serum estradiol did not consistently influence the sex differences in serum calcium and phosphate. In this study, we aimed to evaluate whether the observed sex differences in men and women ≥45 years of age exist at younger age and how serum levels differ between different age groups.

We therefore compared serum calcium and phosphate levels and calcium*phosphate in three age groups of men and women, using three different samples derived from the Hospital Information System (HIS) of the Erasmus MC, with age ranging from 1 - 97 years.

Material and Methods

The data from the study population were derived from the HIS of Erasmus MC. We sampled three different years: 2005, 2010 and 2014. Subjects were included that visited the hospital either in out-patient setting or while being admitted, and in whom serum calcium, phosphate and albumin levels were measured. Per subject only the first serum values measured were included in the analyses. The laboratory values were not coupled to diagnosis, reason for admission or outpatient clinic visit. Only the first serum measurements where serum calcium, serum phosphate and serum albumin were determined at the same time were selected for analyses.

The groups were divided into three different age categories: a group aged 1-17 years old, representing youth; a group aged 18-44 years old, representing adolescence and young adulthood; and a group above ≥45 years. The latter group is based on our previous study where we addressed sex differences in calcium homeostasis (unpublished data) which was done in a population-based cohort study where subjects 45 years or older [7] were included. Furthermore, 45 years is the approximate age when hormonal changes associated with natural menopause first appear [8, 9].

The total number of subjects in the different groups is depicted in Table 1 and included 4074 subjects in 2005, 4708 subjects in 2010 and 6433 subjects in 2014. In the 2014 sample, four subjects had assigned dates of birth on 1-1-1900 since their real date of birth was unknown; therefore these subjects were excluded from the analyses. Since the data were anonymized and there was the possibility of including subjects three times when all samples were merged, we chose to analyse the three samples separately. The Medical Ethical Committee of the Erasmus MC approved the study.

Assay methods

Serum samples from subjects were analyzed at the Department of Clinical Chemistry of Erasmus MC Rotterdam, The Netherlands.

Serum albumin, calcium and inorganic phosphate were measured using the Hitachi 917 Analyzer (Roche, Mannheim, Germany). Calcium*phosphate ($mmol^2/L^2$) product was obtained multiplying the subjects' serum calcium and phosphate levels. Serum calcium corrected for albumin levels was calculated using the formula: calcium (corrected)=calcium measured + 0.02 x (42-albumin measured) [10].

Statistical Analyses

Data from the three different samples were analyzed separately. Sex differences between serum calcium and phosphate were tested in the three different age categories as well as in decades using analysis of covariance (ANCOVA) within a general linear model with either serum calcium, serum phosphate or calcium*phosphate as dependent variables and sex as a fixed factor. Total serum calcium includes: calcium bound to albumin, calcium bound to other proteins and ionized calcium. Moreover, serum albumin levels are lower in critical ill patients and are correlated with disease status. Therefore, data were adjusted for age and serum albumin levels. As a sensitivity analysis, we used the formula as described above in the assay methods, to correct individual serum calcium for their individual albumin levels. Furthermore, to exclude an impact of very high or low albumin levels on serum calcium levels, we performed sensitivity analyses restricting our data only to those subjects with albumin levels within a narrow range (38-42 g/L).

Sex differences in serum calcium corrected for albumin were assessed using ANCOVA within a general linear model with serum calcium corrected for albumin as dependent variable and sex as a fixed factor. Since albumin was part of the formula, we adjusted the corrected serum calcium analyses only for age.

Differences between the age groups in serum calcium levels, corrected serum calcium levels, serum phosphate levels and calcium*phosphate were assessed stratified by sex using ANCOVA in a general linear model with serum calcium, corrected serum calcium, phosphate and calcium*phosphate as dependent variables and age groups as a fixed factor. Analyses were adjusted for albumin, apart from the analysis for corrected serum calcium. The interaction term between sex and age on serum calcium and phosphate was determined using linear regression analyses. Statistical analyses were performed using SPSS (version 23). Statistical significance was defined as a $p < 0.05$.

Potential non-linearity of the associations was tested through the comparison of linear models and natural cubic spline models. We used the STATA package *uvrs* (univariate regression spline), that selects the spline model for the covariate of interest that provides the best prediction for the outcome [11]. When other covariates were added, they were adjusted for linearly. By default, the spline regression applies three spline knots (m=3) which are located in equally spaced centiles of the distribution of the covariate. We ran likelihood ratio tests (LRTs) to compare between models, as linear models (m=0) are nested within spline models. Additionally, models were compared through Akaike Information Criteria (AIC) [12]: the lower, the better fit.

Stata (version 15, College Station TX: Stata Corp LP) was used for spline regression models and plots.

Results

Sample characteristics

For this study, three samples from the HIS were collected. The 2005 sample contained 2235 men with an average age of 48.87 years and 1839 women with an average age of 47.55 years. The 2010 sample contained 2635 men with an average age of 53.25 years and 2073 women with an average age of 52.03 years. The 2014 sample contained 3547 men with an average age of 53.17 years and 2882 women with an average age of 51.38 years.

Sex differences

In subjects 45 years and older, women had higher serum calcium levels compared to men in all three samples (Table 1). As for serum calcium, women ≥45 years of age displayed higher serum phosphate levels compared to men (Table 1). As a consequence, calcium*phosphate was also higher. The age groups 1-17 years and 18-44 years showed no sex difference in serum calcium and phosphate levels

in any of the three samples (Table 1). In Figure 1, serum calcium and phosphate levels are shown per decade in men and women in the three years sampled. In the sample from 2005, there were only ten men and women in the age group above 90, therefore these were not depicted in the Figure. In the 2005 and 2014 samples, a significant sex difference in serum phosphate appeared in the 5th decade, whereas in the 2010 sample this appeared in the 6th decade. In the 2005 sample a significant sex difference appeared for serum calcium levels in the 7th decade, whereas in the 2010 and 2014 samples this difference appeared in the 6th decade.

Sensitivity analyses related to serum albumin levels

Sex differences in albumin levels were present in the age group 18-44 years in all three samples with men having higher albumin levels compared to women. However, in the 2010 and 2014 samples, men ≥45 years had lower serum albumin levels compared to women of the same age. In the youngest age groups in all samples no sex differences were found for serum albumin levels.

After correcting serum calcium for serum albumin levels (according to the formula described in the Material and methods), sex differences in serum calcium levels remained with women having higher corrected serum calcium levels compared to men in the age group above 45 years of age (data not shown).

After restricting our analyses to subjects with albumin levels within the normal range (38-42 g/L), we also found similar results (data not shown).

Age differences in serum calcium and phosphate stratified by sex

Figure 2 depicts serum calcium and phosphate levels per age group stratified by sex.

Men

In all three years samples, men between 1 and 17 years of age had higher serum calcium compared to the age group ≥45 years ($p=4.6x10^{-5}$ in 2005, $p=1.3x10^{-4}$ in 2010 and p=0.006 in 2014); when compared to the age group 18-44 years it was significant in 2005 sample ($p=6.5x10^{-5}$) and in 2010 sample ($p=2.3x10^{-9}$) (Figure 2A).

Serum phosphate levels were higher in the youngest age group compared to the age group 18-44 ($p=2.0x10^{-58}$, $p=9.5x10^{-32}$ and $p=7.9x10^{-50}$ for 2005, 2010 and 2014 samples, respectively) and also when compared to the group of men ≥45 years of age ($p=5.6x10^{-86}$, $p=3.4x10^{-38}$ and $p=1.2x10^{-77}$ for 2005, 2010 and 2014 samples, respectively (Figure 2B). Only in 2014 sample the group of men ≥45 years had significantly higher serum phosphate levels compared to the adult group of 18-44 years (p=0.003) (Figure 2B).

Women

In 2005, 2010 and 2014 samples, women in the age group 1-17 years had significantly higher serum adjusted calcium levels compared to 18-44 years ($p=3.0x10^{-4}$, $p=2.1x10^{-8}$, and $p=1.1x10^{-5}$ for 2005, 2010 and 2014, respectively) (Figure 2C). Women ≥45 years had significantly higher serum calcium levels compared to women in the age group 18-44 years ($p=3.1x10^{-5}$, $p=1.5x10^{-9}$ and $p=6.8x10^{-18}$ for 2005, 2010 and 2014 samples, respectively) (Figure 2C). There was no significant difference in serum calcium levels in women aged 1-17 compared to women ≥45 years (Figure 2C).

Serum phosphate differed significantly between the three different age groups in all samples. The age group 1-17 years had the highest serum phosphate levels whereas the age group 18-44 had lowest serum phosphate levels (Figure 2D).

Non-linearity

We found strong statistical evidence of non-linearity in the associations between calcium and phosphate levels and age across sexes and samples with only one exception: calcium levels in men of the 2010 sample. Results from LRTs and AIC are displayed in Table 2. Sex-stratified spline plots are displayed in Supplementary Figures 1a, 1b and 1c.

Interaction between sex and age on sex differences in serum calcium and phosphate

Interaction analyses of age and sex are shown in Table 3. In all three samples there was a significant association of the sex*age interaction term with serum calcium, corrected serum calcium, serum phosphate and calcium*phosphate. In the three samples, the sex*age interaction terms, age or sex independently, were not consistently associated with serum calcium and phosphate levels.

Discussion

In this study, we found sex differences in serum calcium and phosphate that were age dependent with women above the age of 45 years having higher serum calcium and phosphate levels and a higher calcium*phosphate compared to men. Also, the levels in women above 45 years were higher compared to women < 45 years of age. No sexual dimorphism was found in the younger age groups. Sex differences in serum phosphate appeared on average 10 years before sex differences in serum calcium. Furthermore, there was a significant interaction between age and sex for both serum calcium and phosphate in all samples. Despite the relatively small sex differences in serum calcium and phosphate, our findings may be important in relation to diseases with clear age-related sex difference such as cardiovascular diseases, metabolic syndrome, osteoporosis, and mortality [3, 13-17]. The fact that women with osteoporosis are reported to have an increased risk for cardiovascular disease exemplifies this [18]. Moreover, women with higher coronary artery

calcification scores were found to have lower BMD, an association that is not found in men [19]. A recent study using Mendelian Randomization showed that a genetic predisposition for higher serum calcium was associated with increased coronary artery disease and myocardial infarction [20]. These findings may underlie the sex differences within osteoporosis and cardiovascular diseases. Therefore, understanding the physiological mechanisms that underlie these small differences can aid in the further understanding of these diseases.

There are a few older and mostly small-sized studies that have found, although not always consistently, higher serum calcium levels in elderly women compared to men [13, 21-23]. Several studies have consistently shown that postmenopausal women have higher serum phosphate levels than men of similar age [3, 13, 15, 17, 24, 25]. Furthermore, several studies have shown an age-related decline in serum phosphate levels with sex differences appearing around menopause: women show increases in serum phosphate around 40 years of age, before decreasing again after the age of 60 [14, 26].

This study, as well as a population-based cohort study (Koek *et al*, unpublished data) have shown that there is a clear age-related sex difference with respect to serum calcium and phosphate levels. These sex differences appear to evolve around the age of menopause for serum phosphate and a decade later for serum calcium.

Previously, it has been hypothesized that a fall in estrogens around menopause induces the sex differences in serum calcium and phosphate levels [27, 28]. When a fall in estrogens is hypothesized to cause changes in serum calcium and phosphate levels, one can speculate whether there is an opposite change during growth spurt when estrogen levels increase. Laboratories report different reference values during childhood for serum calcium and phosphate in boys and girls at different ages (emedicine.medscape.com; serum calcium and serum phosphate; reference values) which may indicate that there is a sex difference and potentially a relation between sex-hormones and serum levels of calcium

and phosphate. Several studies have shown serum phosphate levels to be highest in early infancy and to decrease during the course of childhood reaching adult values in late adolescence [29-32]. The higher phosphate levels in childhood are attributed to increased demands for adequate bone growth [33]. In a study of 1984 Krabbe *et al* have shown a relation of serum phosphate but not calcium levels with puberty development.

Serum phosphate levels increase 9 to 12 months before peak height velocity and three months before the first pubic hair, Tanner stage 2 [34]. In our study, we did not find sex differences in serum calcium and phosphate in the youngest age group in any of the samples. Due to the relative small number of subjects in the young groups we were not able to further stratify into smaller age groups in order to assess if serum calcium and phosphate levels changed during different ages in childhood and whether this is sex dependent.

Since the largest differences in sex hormones between boys and girls exist during puberty, assessing the effect of the hormonal surge on serum calcium and phosphate levels will shed light on the relation between hormonal changes and serum calcium and phosphate levels.

Another interesting group of subjects to study the effects of hormones on calcium and phosphate homeostasis would be women during different times in their menstrual cycle, to observe whether hormonal fluctuations influence serum calcium and phosphate levels.

This study was not set up to investigate the molecular pathways behind this age dependent sexual dimorphism in serum calcium and phosphate levels.

Strengths and limitations

The strength of this study is the availability of data in a large number of men and women in the three samples evaluated.

Since we analyzed serum levels from a database of a hospital laboratory, there may be a bias in the analyses because of underlying medical conditions that prompted the ordering of measurements of serum calcium and phosphate levels in these subjects. Since we had no information on these conditions, we were not able to take into account or exclude diseases that influence calcium or phosphate homeostasis.

Therefore, one should be cautious with extrapolation of these results to a healthy population. Despite these flaws in design, the differences between the sexes in older subjects were present in all three samples and are in line with our previous study, where we assessed serum calcium and phosphate levels in an aging population study (Koek *et al*., unpublished data). Other limitations of our study are the lack of data on hormone levels such as estradiol and testosterone and on other hormones that are more directly linked with calcium and phosphate metabolism including vitamin D, PTH and FGF23 levels that could also underlie the observed sex differences in serum calcium and phosphate levels.

Conclusions

The results of this study showed that there is a consistent sex difference in serum calcium and phosphate levels and calcium*phosphate and that this is age-dependent, with women ≥45 years of age having significantly higher serum levels of calcium, phosphate and calcium*phosphate compared to men of similar age and compared to women < 45 year of age. There were no sex differences in the age groups < 45 years. Furthermore, women but not men ≥45 years have higher serum calcium and phosphate levels compared to the group < 45 years, suggesting that a decline in sex steroid levels after menopause is responsible for these sex differences. These findings may be of relevance for increased incidence of CVD in women after menopause.

Table 1: Calcium, phosphate and albumin levels in men and women in 3 different age groups in 2005, 2010 and 2014. Values are depicted as means with 95% confidence intervals between brackets.

	1-17 years			18-44 years			≥45 years		
2005	Men	Women	P-AN(C)OVA	Men	Women	P-AN(C)OVA	Men	Women	P-AN(C)OVA
Number	249	225		540	510		1446	1104	
Age (years)	9.29 (8.64-9.94)	9.67 (9.04-10.29)	0.42	32.85 (32.19-33.52)	33.20 (32.50-33.90)	0.48	61.66 (61.17-62.16)	61.89 (61.27-62.52)	0.57
Albumin (g/l)	43.02 (42.24-43.79)	42.65 (41.70-43.60)	0.24	42.40 (41.87-42.94)	41.43 (40.92-41.93)	0.009	40.55 (40.23-40.87)	40.70 (40.35-41.06)	0.48
Calcium (mmol/l)	2.409 (2.382-2.437)	2.398 (2.373-2.424)	0.98	2.348 (2.330-2.365)	2.336 (2.320-2.351)	0.36	2.321 (2.312-2.331)	2.352 (2.341-2.363)	$3.6x10^{-7}$
Calcium corrected (mmol/l)	2.389 (2.369-2.410)	2.385 (2.369-2.402)	0.87	2.339 (2.326-2.353)	2.347 (2.336-2.359)	0.38	2.350 (2.344-2.357)	2.378 (2.370-2.387)	$3.6x10^{-7}$
Phosphate (mmol/l)	1.554 (1.515-1.592)	1.546 (1.494-1.598)	0.96	1.115 (1.087-1.144)	1.111 (1.084-1.138)	0.77	1.093 (1.076-1.110)	1.167 (1.151-1.183)	$1.7x10^{-9}$
Calcium* phosphate ($mmol^2/l^2$)	3.750 (3.647-3.853)	3.721 (3.593-3.850)	0.87	2.613 (2.547-2.679)	2.589 (2.527-2.650)	0.91	2.528 (2.490-2.567)	2.747 (2.708-2.787)	$2.1x10^{-14}$
2010									
Number	157	119		591	580		1887	1374	
Age (years)	9.36 (8.54-10.17)	10.67 (9.66-11.68)	0.04	33.64 (32.98-34.30)	33.37 (32.73-34.01)	0.54	63.06 (62.57-63.49)	63.48 (62.90-64.07)	0.23

Albumin (g/l)	41.94 (40.78-43.09)	41.78 (40.54-43.02)	0.76	42.73 (42.10-43.35)	41.70 (41.15-42.25)	0.013	40.22 (39.89-40.56)	40.82 (40.46-41.19)	0.012
Calcium (mmol/l)	2.337 (2.307-2.367)	2.332 (2.302-2.362)	0.60	2.288 (2.273-2.303)	2.259 (2.244-2.273)	0.19	2.247 (2.236-2.257)	2.288 (2.277-2.298)	$2.0x10^{-6}$
Calcium corrected (mmol/l)	2.336 (2.286-2.386)	2.328 (2.279-2.377)	0.95	2.303 (2.276-2.329)	2.253 (2.229-2.277)	0.005	2.211 (2.196-2.227)	2.264 (2.248-2.280)	$3.0x10^{-6}$
Phosphate (mmol/l)	1.434 (1.387-1.481)	1.393 (1.308-1.479)	0.91	1.071 (1.044-1.098)	1.086 (1.064-1.109)	0.61	1.069 (1.053-1.085)	1.136 (1.121-1.152)	$1.4 x10^{-9}$
Calcium* phosphate (mmol2/l2)	3.362 (3.240-3.485)	3.258 (3.069-3.456)	0.99	2.437 (2.378-2.496)	2.451 (2.400-2.503)	0.57	2.388 (2.354-2.421)	2.598 (2.563-2.634)	$7.6x10^{-16}$
2014									
Number	296	254		708	687		2543	1941	
Age (years)	9.61 (9.01-10.21)	10.66 (10.03-11.29)	0.013	33.40 (32.82-33.98)	32.98 (32.40-33.57)	0.33	63.74 (63.36-64.13)	63.22 (62.76-63.69)	0.093
Albumin (g/l)	40.51 (39.65-41.37)	41.05 (40.10-41.99)	0.57	42.64 (42.11-43.16)	40.91 (40.44-41.39)	$2.0x10^{-6}$	38.95 (38.67-39.23)	39.92 (39.61-40.23)	$2.1x10^{-5}$
Calcium (mmol/l)	2.338 (2.316-2.359)	2.347 (2.322-2.373)	0.60	2.345 (2.331-2.355)	2.300 (2.287-2.341)	0.13	2.283 (2.275-2.291)	2.334 (2.325-2.343)	$1.0x10^{-12}$
Calcium corrected (mmol/l)	2.368 (2.353-2.382)	2.366 (2.347-2.386)	0.64	2.332 (2.323-2.342)	2.322 (2.313-2.332)	0.11	2.344 (2.338-2.350)	2.376 (2.369-2.382)	$5.7x10^{-13}$
Phosphate (mmol/l)	1.412 (1.377-1.446)	1.366 (1.323-1.409)	0.33	1.051 (1.027-1.076)	1.043 (1.022-1.064)	0.16	1.044 (1.031-1.057)	1.115 (1.102-1.128)	$3.2x10^{-17}$
Calcium* phosphate ($mmol^2/l^2$)	3.310 (3.224-3.396)	3.203 (3.104-3.301)	0.28	2.453 (2.340-2.506)	2.399 (2.350-2.499)	0.20	2.369 (2.341-2.396)	2.594 (2.565-2.623)	$8.3x10^{-27}$

Table 2. Results of comparison between linear models and spline models

	LRT	AIC linear model	AIC spline model
Sample: 2005			
Ca levels in men	0.002	*-1122*	*-1131*
Ca levels in women	<0.001	*-902*	*-920*
P levels in men	<0.001	1559	1356
P levels in women	<0.001	1017	788
Sample: 2010			
Ca levels in men	0.175	*-443*	*-443*
Ca levels in women	<0.001	*-895*	*-926*
P levels in men	<0.001	2120	1940
P levels in women	<0.001	1037	857
Sample: 2014			
Ca levels in men	0.012	*-1304*	*-1309*
Ca levels in women	<0.001	*-1056*	*-1082*
P levels in men	<0.001	2508	2165
P levels in women	<0.001	1633	1217

LRT: likelihood ratio test; AIC: Akaike information criteria. Italic font: note the negative values: the lower, the better fit.

Table 3: Summary of linear regression analyses with in the model selection for serum calcium, serum phosphate, calcium*phosphate and corrected serum calcium levels

Parameters	Variables in the model	Regression coefficient (95% CI)	Standardized Beta	P Value	R2 of the final Model
2005					
Serum Calcium (mmol/l)	Sex	-0,0148 (-0.036/0.0069)	-0.039	0.18	
	Age	-0.000404 (-0,00069/-0.00012)	-0.044	0.005	
	Albumin	0.0204 (0.0197/0.0211)	0.671	$0.0x10^{0}$	
	Sex*age interaction	0.000686 (0.000273/0.00110)	0.100	0.001	
Serum Phosphate (mmol/l)	Sex	-0.044789 (-0.096496/0.006919)	-0.064	0.09	
	Age	-0.005297 (-0.00597/-0.00462)	-0.318	1.2×10^{-51}	0.2078
	Albumin	-0.00112(-0.00278/0.00054)	-0.020	0.19	
	Sex*age interaction	0.00181 (0.00082/0.00279)	0.144	3.3×10^{-4}	
Calcium*phosphate (mmol2/l2)	Sex	-0.12066 (-0.24159/0.00027)	-0.072	0.05	
	Age	-0.01332 (-0.01490/-0.01174)	-0.334	3.6×10^{-59}	0.123
	Albumin	0.02074 (0.01686/0.02462)	0.156	2.4×10^{-25}	
	Sex*age interaction	0.00512 (0.00282/0.00742)	0.170	1.4×10^{-5}	
Corrected serum calcium (mmol/l)	Sex	-0.01510 (-0.03680/-0.006595)	-0.054	0.17	
	Age	-0.00043 (-0.00071/-0.000144)	-0.063	0.0031	0.007
	Sex*age interaction	0.00069 (0.00028/0.00111)	0.136	0.0011	

2010					
Serum Calcium (mmol/l)	Sex	-0.02823 (-0.05511/-0.00134)	-0.066	0.04	
	Age	-0.00036 (-0.00069/-0.00004)	-0.033	0.03	0.423
	Albumin	0.01882 (0.01818/0.01946)	0.647	0.0×10^{0}	
	Sex*age interaction	0.000843 (0.00036/0.00132)	0.115	5.8×10^{-4}	
Serum Phosphate (mmol/l)	Sex	-0.04493 (-0.10047/0.01061)	-0.066	0.113	
	Age	-0.003133 (-0.00381/-0.00246)	-0.178	1.4×10^{-19}	
	Albumin	-0.00583 (-0.00714/-0.00451)	-0.126	5.3×10^{-18}	0.036
	Sex*age interaction	0.001699 (0.00071/0.00269)	0.081	7.9×10^{-4}	
Calcium*phosphate ($mmol^2/l^2$)	Sex	-0.13055 (-0.25391/-0.00718)	-0.086	0.04	
	Age	-0.00781(-0.00931/-0.00631)	-0.199	3.7×10^{-24}	0.043
	Albumin	0.00865 (0.00572/0.01157)	0.084	7.2×10^{-9}	
	Sex*age interaction	0.00494 (0.00274/0.00715)	0.190	1.1×10^{-5}	
Corrected serum calcium (mmol/l)	Sex	-0.05961 (-0.11353/-0.00570)	-0.091	0.03	
	Age	-0.00271 (-0.00336/-0.00206)	-0.160	4.8×10^{-16}	0.017
	Sex*age interaction	0.00156 (0.00060/0.00253)	0.139	0.002	
2014					
Serum Calcium (mmol/l)	Sex	-0.03429 (-0.05360/-0.01497)	-0.085	5.0×10^{-4}	
	Age	-0.000223 (-0.00046/0.00001)	-0.022	0.06	0.508
	Albumin	0.02000 (0.01951/0.02049)	0.709	0.0×10^{-0}	
	Sex*age interaction	0.00101 (0.00067/0.00136)	0.146	8.5×10^{-9}	

Serum Phosphate (mmol/l)	Sex	-0.06262 (-0.10594/-0.01931)	-0.096	0.005	
	Age	-0.00363 (-0.004152/-0.00310)	-0.225	6.1×10^{-41}	0.051
	Albumin	-0.00692 (-0.00802/-0.00582)	-0.152	1.4×10^{-34}	
	Sex*age interaction	0.00201 (0.00123/0.00278)	0.178	3.8×10^{-7}	
Calcium*phosphate ($mmol^2/l^2$)	Sex	-0.17317 (-0.27049/-0.07586)	-0.118	4.9×10^{-4}	
	Age	-0.00882 (-0.01000/-0.00763)	-0.243	1.5×10^{-47}	0.053
	Albumin	0.00688 (0.00441/ 0.00935)	0.067	4.9×10^{-8}	
	Sex*age interaction	0.00568 (0.00394/0.00742)	0.225	1.7×10^{-10}	
Corrected serum calcium (mmol/l)	Sex	-0.03428 (-0.05359/-0.01498)	-0.120	0.001	
	Age	-0.00022 (-0.00046/0.00001)	-0.032	0.06	0.010
	Sex*age interaction	0.00102 (0.00067/0.00136)	0.206	8.3×10^{-9}	

Figure 1

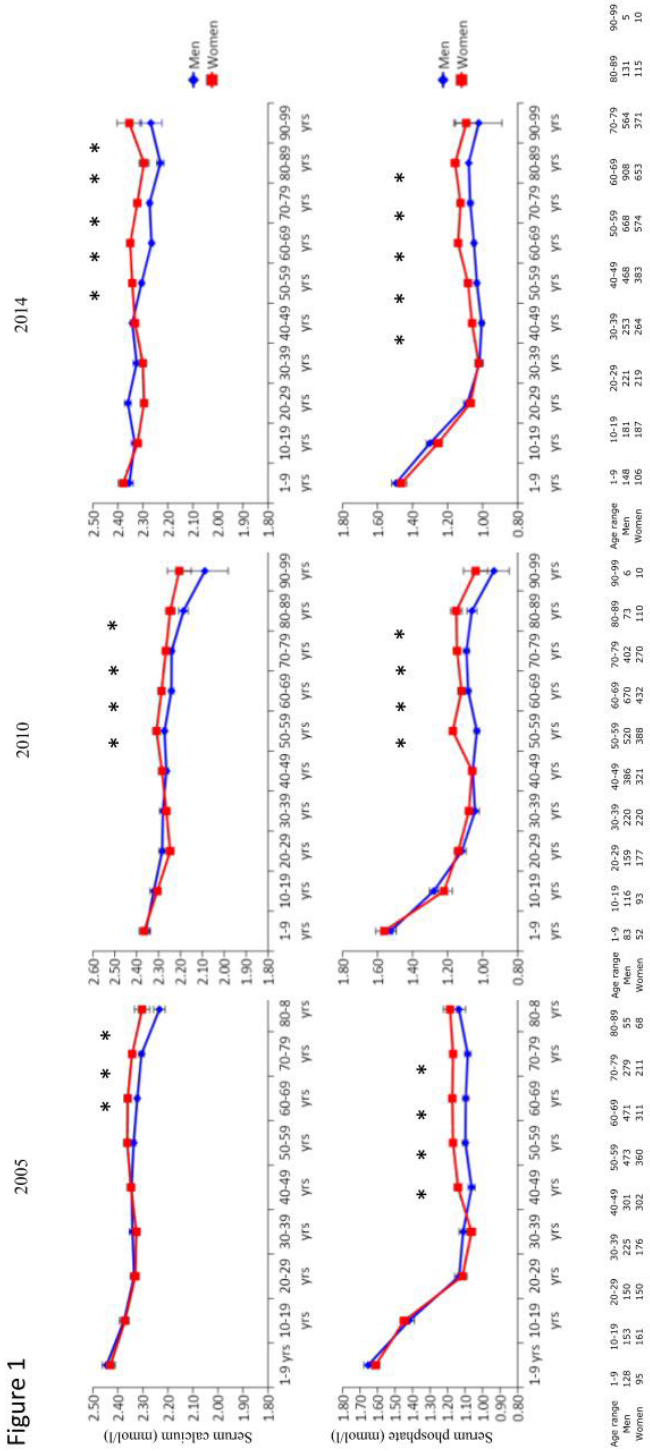

2005
2010
2014
Men
Women
Serum calcium (mmol/l)
Serum phosphate (mmol/l)
1-9 yrs
10-19 yrs
20-29 yrs
30-39 yrs
40-49 yrs
50-59 yrs
60-69 yrs
70-79 yrs
80-8
80-89 yrs
90-99 yrs
Age range 1-9 10-19 20-29 30-39 40-49 50-59 60-69 70-79 80-89
Men 128 153 150 225 301 473 471 279 55
Women 95 161 150 176 302 360 311 211 68
Age range 1-9 10-19 20-29 30-39 40-49 50-59 60-69 70-79 80-89 90-99
Men 83 116 159 220 386 520 670 402 73 6
Women 52 93 177 220 321 388 432 270 110 10
Age range 1-9 10-19 20-29 30-39 40-49 50-59 60-69 70-79 80-89 90-99
Men 148 181 221 253 468 668 908 564 131 5
Women 106 187 219 264 383 574 653 371 115 10

Figure 2A

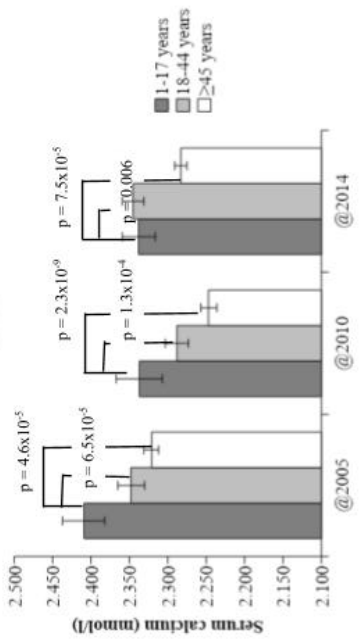


Figure 2B

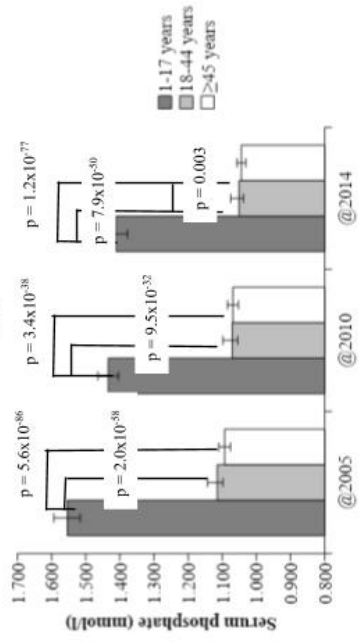


Figure 2C

Figure 2D

References

1. **Barbagallo M., et al**. Effects of aging on serum ionized and cytosolic free calcium: relation to hypertension and diabetes. *Hypertension* 1999; 34(4 Pt 2): p.902-6.
2. **Jorde R., et al**. Serum calcium and cardiovascular risk factors and diseases: the Tromso study. *Hypertension* 1999; 34(3): p. 484-90.
3. **Onufrak S.J., et al**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009; 169(1): p. 67-77.
4. **Tohno Y., et al**. Gender difference in accumulation of calcium and phosphorus in the left coronary arteries of Thais. *Biol Trace Elem Res* 2011; 144(1-3): p. 17-26.
5. **Appelman Y., et al**. Sex differences in cardiovascular risk factors and disease prevention. *Atherosclerosis* 2015.
6. **McCloskey E.V., et al**. The assessment of vertebral deformity: a method for use in population studies and clinical trials. *Osteoporos Int* 1993; 3(3): p. 138-47.
7. **Ikram M.A., et al**. The Rotterdam Study: 2018 update on objectives, design and main results. *Eur J Epidemiol* 2017; 32(9): p. 807-850.
8. **Gold E.B**. The timing of the age at which natural menopause occurs. *Obstet Gynecol Clin North Am* 2011; 38(3): p. 425-40.
9. **Trevoux R., et al**. Endometrium and plasma hormone profile in the peri-menopause and post-menopause. *Maturitas* 1986; 8(4): p. 309-26.
10. **Payne R.B., et al**. Interpretation of serum calcium in patients with abnormal serum proteins. *Br Med J* 1973; 4(5893): p. 643-6.
11. **Royston P S.W**. Multivariable modeling with cubic regression splines: a principled approach. *The Stata Journal* 2007; 7(1): p. 45-70.
12. **Akaike H**. An information criterion (AIC). *Math Sci* 1976; (14): p. 5-9.
13. **Keating F.R., Jr., et al**. The relation of age and sex to distribution of values in healthy adults of serum calcium, inorganic phosphorus, magnesium, alkaline phosphatase, total proteins, albumin, and blood urea. *J Lab Clin Med*

1969; 73(5): p. 825-34.

14. **Cirillo M., C. Ciacci, and N.G. De Santo.** Age, renal tubular phosphate reabsorption, and serum phosphate levels in adults. *N Engl J Med* 2008; 359(8): p.864-6.

15. **de Boer I.H., T.C. Rue, and B. Kestenbaum**. Serum phosphorus concentrations in the third National Health and Nutrition Examination Survey (NHANES III). *Am J Kidney Dis* 2009; 53(3): p. 399-407.

16. **Dhingra R., et al**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007; 167(9): p. 879-85.

17. **Tonelli M., et al**. Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005; 112(17): p. 2627-33.

18. **Tanko L.B., et al**. Relationship between osteoporosis and cardiovascular disease in postmenopausal women. *J Bone Miner Res* 2005; 20(11): p. 1912-20.

19. **Campos-Obando N. et al**. Bone health and coronary artery calcification: The Rotterdam Study. *Atherosclerosis* 2015; 241(1): p. 278-83.

20. **Larsson S.C., S. Burgess and K. Michaelsson**. Association of Genetic Variants Related to Serum Calcium Levels With Coronary Artery Disease and Myocardial Infarction. *JAMA* 2017; 318(4): p. 371-380.

21. **Lindgarde F**. Potentiometric determination of serum ionized calcium in a normal human population. *Clin Chim Acta* 1972; 40(2): p. 477-84.

22. **Nordin B.E., et al**. Biochemical variables in pre- and postmenopausal women: reconciling the calcium and estrogen hypotheses. *Osteoporos Int* 1999; 9(4): p. 351-7.

23. **Roof B.S., et al**. Serum parathyroid hormone levels and serum calcium levels from birth to senescence. *Mech Ageing Dev* 1976; 5(4): p. 289-304.

24. **Boyd J.L., M.E. Delost, and J.P. Holcomb**. Calcium, phosphorus, and alkaline phosphatase values of elderly subjects. *Clin Lab Sci* 1998; 11(4): p. 223-7.

25. **Foley R.N., et al**. Calcium-phosphate levels and cardiovascular disease in community-dwelling adults: the Atherosclerosis Risk in Communities (ARIC) Study. *Am Heart J* 2008; 156(3): p. 556-63.

26. **Zhang D., et al**. Effects of sex and postmenopausal estrogen use on serum phosphorus levels: a cross-sectional study of the National Health and Nutrition Examination Survey (NHANES) 2003-2006. *Am J Kidney Dis* 2014; 63(2): p. 198-205.

27. **Nordin B.E., et al**. Evidence for a renal calcium leak in postmenopausal women. *J Clin Endocrinol Metab* 1991; 72(2): p. 401-7.

28. **Young M.M. and B.E**. **Nordin**. Calcium metabolism and the menopause. *Proc R Soc Med* 1967; 60(11 Part 1): p. 1137-8.

29. **Brodehl J., K. Gellissen, and H.P**. **Weber**. Postnatal development of tubular phosphate reabsorption. *Clin Nephrol* 1982; 17(4): p. 163-71.

30. **Burritt M.F., et al.** Pediatric reference intervals for 19 biologic variables in healthy children. *Mayo Clin Proc* 1990; 65(3): p. 329-36.

31. **Arnaud S.B., et al**. Serum parathyroid hormone and blood minerals: interrelationships in normal children. *Pediatr Res* 1973; 7(5): p. 485-93.

32. **Greenberg B.G., R.W. Winters, and J.B. Graham.** The normal range of serum inorganic phosphorus and its utility as a discriminant in the diagnosis of congenital hypophosphatemia. *J Clin Endocrinol Metab* 1960; 20: p. 364-79.

33. **Penido M. and U.S. Alon**. Phosphate homeostasis and its role in bone health. *Pediatr Nephrol* 2012; 27(11): p. 2039-48.

34. **Krabbe S. and C**. **Christiansen**. Longitudinal study of calcium metabolism in male puberty. I. Bone mineral content, and serum levels of alkaline phosphatase, phosphate and calcium. *Acta Paediatr Scand* 1984; 73(6): p. 745-9.

9

PLS3 Mutations in X-Linked Osteoporosis with Fractures

PLS3 Mutations in X-Linked Osteoporosis with Fractures

Authors:

Fleur S. van Dijk*, M. Carola Zillikens*,
Dimitra Micha*, Markus Riessland*, Carlo L.M. Marcelis,
Christine E. de Die-Smulders, Janine Milbradt,
Anton A. Franken, Arjan J. Harsevoort, Klaske D. Lichtenbelt,
Hans E. Pruijs, M. Estela Rubio-Gozalbo, Rolf Zwertbroek,
Youssef Moutaouakil, Jaqueline Egthuijsen,
Matthias Hammerschmidt, Renate Bijman, Cor M. Semeins,
Astrid D. Bakker, Vincent Everts, Jenneke Klein-Nulend,
Natalia Campos-Obando, Albert Hofman,
Gerard J. te Meerman, Annemieke J.M.H. Verkerk,
André G. Uitterlinden, Alessandra Maugeri, Erik A. Sistermans,
Quinten Waisfisz, Hanne Meijers-Heijboer, Brunhilde Wirth,
Marleen E.H. Simon, and Gerard Pals

*These authors contributed equally to this work

Status:

Published in *N Eng J Med* 2013;369:1529-36

Summary

Plastin 3 (PLS3), a protein involved in the formation of filamentous actin (F-actin) bundles, appears to be important in human bone health, on the basis of pathogenic variants in PLS3 in five families with X-linked osteoporosis and osteoporotic fractures that we report here. The bone-regulatory properties of PLS3 were supported by in vivo analyses in zebrafish. Furthermore, in an additional five families (described in less detail) referred for diagnosis or ruling out of osteogenesis imperfecta type I, a rare variant (rs140121121) in PLS3 was found. This variant was also associated with a risk of fracture among elderly heterozygous women that was two times as high as that among noncarriers, which indicates that genetic variation in PLS3 is a novel etiologic factor involved in common, multifactorial osteoporosis.

Osteoporosis is a prevalent disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, which results in bone fragility and fractures.[1] It is diagnosed clinically and often confirmed by measuring bone mineral density (BMD).[1,2] An understanding of the causes of osteoporosis is important for its prevention, diagnosis, and treatment. The investigation of rare mendelian disorders with decreased BMD as a key diagnostic feature constitutes a strategy for identifying genetic determinants of osteoporosis.[3-7]

We identified families with X-linked osteoporosis and fractures among patients with negative tests for the genes encoding collagen type Iα1 and type Iα2 (*COL1A1* and *COL1A2*, respectively) who had been referred to us for diagnosis or ruling out of osteogenesis imperfecta type I. Osteoporosis with fractures as an X-linked trait has been reported by Sillence.[8] We now report data from five families with X-linked osteoporosis and fractures related to pathogenic variants in the gene for plastin 3 (*PLS3*), provide functional evidence that PLS3 is a bone-regulatory protein, and describe a rare variant or single-nucleotide polymorphism (SNP) associated with decreased BMD and an increased risk of fracture among heterozygous women in the general population.

Methods

Families

The pedigrees and clinical characteristics of Families 1 through 5 are provided in Figure 1 and Table 1, and Figure S1 and Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Five additional families, designated Families 6 through 10, were also included in the study and are mentioned in less detail (Fig. S2 and Table S2 in the Supplementary Appendix).

Genetic studies

Three patients with osteoporosis and fractures from Family 1 (Patients 1.III-2, 1.IV-3, and 1.IV-7) underwent X-linked whole-exome sequencing.[9,10] We then performed Sanger sequencing of all *PLS3* exons in 95 affected male patients without *COL1A1* or *COL1A2* mutations who had been referred for diagnosis or ruling out of osteogenesis imperfecta type I. Complementary DNA (cDNA) analysis was performed in Patients 1.III-2 and 3.II-1 and the index patient from Family 9. Linkage analysis was conducted in Families 1 and 2. Methodologic and other details of the studies performed are described in the Supplementary Appendix.

Epidemiologic studies

The rs140121121 SNP was genotyped in three cohorts (RS-I, RS-II, and RS-III) of the prospective, population-based Rotterdam Study, which has analyzed, among other topics, the association of genetic factors with BMD and incident fractures in Dutch men and women 45 years of age or older.[11] Details of these studies are provided in the Supplementary Appendix.

Functional studies

Electrophoresis of type I collagen and Western blot analysis for PLS3 were performed in affected Patients 1.III-2, 1.IV-2, 1.IV-7, 1.IV-8, 3.II-1, and 4.II-1 and the index patients from Families 7 and 9. PLS3, belonging to the family of plastins, is involved in the formation of F-actin bundles.[12] The effect of PLS3 deficiency on F-actin cytoskeleton was investigated in dermal fibroblasts with the use of immunofluorescence microscopy. We hypothesized that PLS3 may be involved in mechanosensing of osteocytes. Mechanical loading in the form of fluid shear stress increases the production of nitric oxide in bone cells,[13] periodontal ligament, and gingival fibroblasts.[14]

In the absence of bone tissue from patients, we investigated the response to fluid shear stress of dermal fibroblasts from six patients with *PLS3* mutations, as compared with three patients with molecularly confirmed osteogenesis imperfecta type I and eight controls. To characterize the effect of loss of PLS3 on bone morphology, we performed morpholino-mediated knockdown of the zebrafish homologue (National Center for Biotechnology Information [NCBI] Reference Sequence [RefSeq], NM_001002326.1). Since cartilaginous pharyngeal arches are the earliest formed craniofacial skeletal elements, we used a *col1a1:eGFP* (enhanced green fluorescent protein under the control of a col1α1-promoter) transgenic zebrafish line to monitor skeletal development.[15] Details of these studies are provided in the Supplementary Appendix.

Results

Genetic studies

Identification of Pathogenic Variants in PLS3

We discovered a single deleterious hemizygous frameshift, c.235delT;p.(Tyr79Ilefs*6), in exon 3 of *PLS3* (NCBI Reference Sequence, NM_005032.5; Mendelian Inheritance in Man number, 300131; chromosome-map location, Xq23) in Patients 1.III-2, 1.IV-3, and 1.IV-7 (Fig. S3A through S3F in the Supplementary Appendix). Sanger sequencing confirmed the presence of this variant in six affected male patients and its absence in one unaffected male patient (Fig. 1).

Sanger sequencing of all *PLS3* exons in 95 affected male patients without *COL1A1* or *COL1A2* mutations yielded four pathogenic variants in Families 2 through 5 (Fig. 1). In Family 2, a nonsense mutation, c.1471C→T;p.(Gln491*), in exon 13 was identified in Patients 2.III-3 and 2.III-7. In Families 3, 4, and 5, three pathogenic variants were identified: a splice-site variant, c.748+1G→A, in exon 7 (in Patient 3.II-1); an insertion, c.759_760insAAT;p.(Ala253_Leu254insAsn), in exon 8 (in actin-binding domain 1, conserved from human down to tetraodon) (in Patient

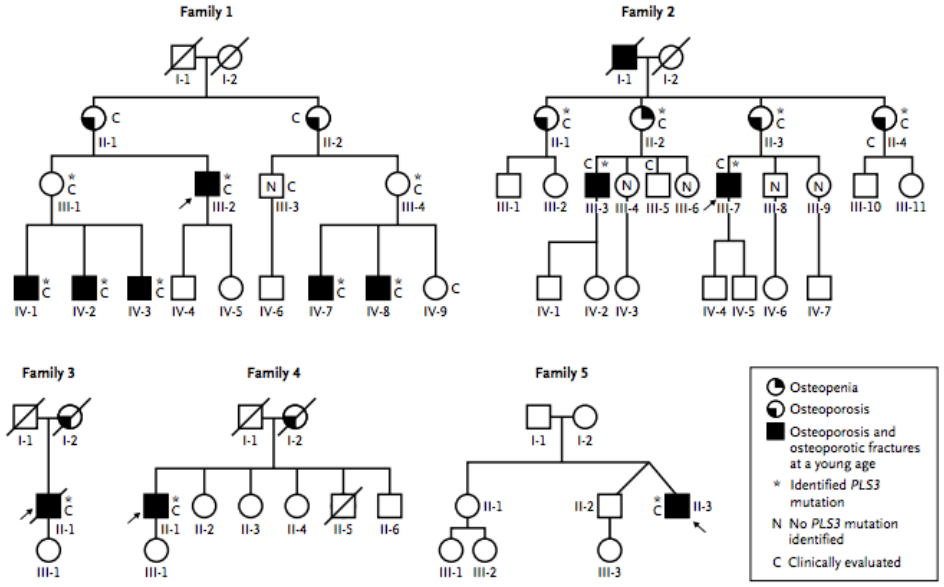
4.II-1); and a frameshift variant, c.1647delC;p.(Ser550Alafs*9), in exon 15 (in Patient 5.II-3). To our knowledge, none of these variants are described in current databases of human sequence variants: data from the 1000 Genomes Project, the Single Nucleotide Polymorphism database (dbSNP, build 137), or data from the GO Exome Sequencing Project (ESP) of the National Heart, Lung, and Blood Institute (http://evs.gs.washington.edu/EVS).

In addition, a c.321T→A variant in exon 4b (Fig. S3F in the Supplementary Appendix), listed in dbSNP as rs140121121, was identified in 5 patients (from Families 6 through 10) among the 95 male patients referred to us for possible osteogenesis imperfecta type I (allele frequency, 0.05) (Table S2A in the Supplementary Appendix). For this rare variant, the allele frequency was 0.01 among 1872 men in the ESP and 0.02 among the 5189 men in the Rotterdam Study, results that differ significantly from the frequency among our 95 male patients (P=0.006 and P=0.04 by two-tailed Fisher's exact test for the two comparisons, respectively).

cDNA Analysis

In Family 3 (Patient 3.II-1), a partial skipping of exon 7 and use of a cryptic splice site, c.748+36, was detected (Fig. S4A and S4B in the Supplementary Appendix).

Figure 1. Pedigrees of Families 1 through 5 with Mutations in the Gene for Plastin 3 (*PLS3*).



We identified five pathogenic variants in *PLS3* in hemizygous male family members in Families 1 through 5, associated with osteoporosis and osteoporotic fractures of the axial and appendicular skeleton developing in childhood. Patient 1.IV-1 had a mild phenotype with a forearm fracture at the age of 8 years, mild osteopenia at the age of 13 years, and two vertebral compression fractures diagnosed at the age of 21 years. Patient 4.II-1 received a diagnosis of osteoporosis and osteoporotic fractures in adulthood. Physical examination did not reveal abnormalities, and specifically, no extraskeletal features of osteogenesis imperfecta were observed. Apart from a waddling gait in two brothers (Patients 1.IV-7 and 1.IV-8), which disappeared for unknown reasons, no neuromuscular abnormalities were reported. Available radiographs did not show abnormalities in bone size or shape. Serum calcium and phosphate levels were normal in all affected male family members, as was urinary calcium excretion, which was measured in several of the affected patients. No consistent decrease or increase in bone-turnover markers was observed. The clinical picture in heterozygous female members in Families 1 and 2 was varied, ranging from normal bone mineral density and an absence of fractures to early-onset osteoporosis. Osteopenia and osteoporosis were diagnosed by means of dual-energy radiographic absorptiometry according to World Health Organization criteria. Squares represent male family members, circles female family members, and slashes deceased family members. Arrows indicate the probands. Additional clinical details from Families 1 through 5 are available in Tables S1, S2, and S3 in the Supplementary Appendix.

Table 1. Clinical and Bone-Densitometry Findings in 11 Male Patients from Five Families with a Pathogenic Variant in the Gene for Plastin 3 (*PLS3*).*

Patient†	Before Therapy				After Therapy‡				Low-Impact Peripheral Fractures	Multiple Vertebral Fractures	Other Clinical Findings§
	Age	BMD z Score			Age	BMD z Score					
		lumbar spine	*femoral neck*	*total body*		*lumbar spine*	*femoral neck*	*total body*			
	yr				*yr*				*no.*		
1.III-2	32	−5.5	−3.4	NA	40	−4.6	−3.1	NA	13	Yes	None
1.IV-1	13	−1.2	NA	−1.5	NT	NT	NT	NT	1	No	None
1.IV-1	21	−1.1	−0.8	−0.8	NT	NT	NT	NT	1	Yes	None
1.IV-2	10	−2.1	NA	−3.0	17	0.9	NA	−0.7	6	No	Acute lymphatic leukemia
1.IV-3	4	−3.2	NA	−3.6	10	−1.2	NA	−1.4	1	No	None
1.IV-7	6	−3.7	NA	−4.6	14	0.7	NA	−1.1	17	No	Patent ductus arteriosus and, in childhood, waddling gait
1.IV-8	10	−2.4	NA	−3.3	12	−1.1	NA	−1.9	Multiple	No	Epilepsy and, in childhood, waddling gait
2.III-3	36	−2.8	−2.3	NA	NA	NA	NA	NA	5	No	None
2.III-7	34	−3.4	−3.4	NA	NA	NA	NA	NA	13	Yes	None
3.II-1	NA	NA	NA	NA	47	−3.75	−2.5	NA	Multiple	Yes	Alcohol abuse

3.II-1	NA	NA	NA	NA	62	NA	−1.0	NA	Multiple	Yes	Esophageal carcinoma
4.II-1	54	−2.5	−0.7	NA	61	−1.0	−0.6	NA	1	Yes	None
5.II-3	41	−2.8	NA	NA	NA	NA	NA	NA	10	Yes	None

* Hemizygous male family members were considered to be affected if the bone mineral density (BMD) z score was below −2.0 SD or the T score was below −2.5 SD. They were also considered to be affected if they had multiple vertebral compression fractures and if secondary causes of osteoporosis had been considered and ruled out on the basis of the medical history, physical examination, protein electrophoresis, and measurements of serum levels of calcium, albumin, phosphate, creatinine, 25-hydroxyvitamin D, thyrotropin, and testosterone; in several patients, the measurement of urinary calcium excretion was also used. NA denotes not available, and NT not treated.

† Two patients (Patients 1.IV-1 and 3.II-1) underwent more than one evaluation.

‡ Therapy refers to bisphosphonate treatment (pamidronate, alendronate, zoledronate, or risedronate), which was initiated in almost all affected patients and was associated with a favorable outcome.

§ No specific extraskeletal features of osteogenesis imperfecta, such as blue sclerae, hearing loss, or dentinogenesis imperfecta, were noted. Patients 1.IV-3, 1.IV-7, and 1.IV-8 had joint hypermobility.

Table 2. Sex-Combined Fracture Risk in Two Rotterdam Study Cohorts, According to rs140121121 Genotype.*

Cohort†	Genotype 0	Genotype 1	Genotype 1 vs. Genotype 0		Genotype 2	Genotype 2 vs. Genotype 0	
	Persons with Fracture	Persons with Fracture	Odds Ratio (95% CI)	P Value	Persons with Fracture	Odds Ratio (95% CI)	P Value
	no./total no.				*no./total no.*		
RS-I	1474/6017	44/118	1.74 (1.19–2.55)	0.004	11/58	0.71 (0.37–1.38)	0.31
RS-II	222/2375	10/43	2.99 (1.44–6.20)	0.003	0/27	NA	—
Both cohorts	1696/8392	54/161	1.95 (1.39–2.74)	<0.001	11/85	NA	—

* Genotype 0 was defined as T in men and TT in women, genotype 1 as TA in women, and genotype 2 as A in men and AA in women.

† The cohorts were from the prospective, population-based Rotterdam Study involving analyses of the associations among genetic factors, BMD, and incident fractures in Dutch men and women 45 years of age or older.[11]

Use of this cryptic splice site leads to an in-frame insertion of 36 nucleotides in the messenger RNA (mRNA) and an insertion of 12 amino acids in PLS3: p.(Glu249_Ala250ins12) (NCBI RefSeq, NP_001129497.1) in the highly conserved actin-binding domain 1. The in-frame insertion is consistent with the results of Western blot analysis, which showed a detectable but reduced PLS3 level (the difference in molecular weight of the proteins of approximately 1 kD is not detectable on Western blot testing) (Fig. S5 in the Supplementary Appendix). In fibroblasts from Family 9 with the c.321T→A exon 4 variant, cDNA with primers for exons 4 (forward) and 7 (reverse) was normal.

Linkage Analysis

The combined LOD score in Families 1 and 2 was 3.40 (2.35 in Family 1 and 1.05 in Family 2). Thus, it is very likely that the identified variants in *PLS3* were causative.

Epidemiologic Studies

The minor allele frequencies of the rs140121121 SNP in men and women, respectively, in the RS-I, RS-II, and RS-III cohorts were 0.022 and 0.016, 0.024 and 0.017, and 0.012 and 0.016. To investigate the relationship of this variant with fracture risk, we performed sex-combined analyses for X- linked inheritance with adjustment for age and bodymass index but not sex, treating men as homozygous women.[16]

In the two cohorts with fracture information (RS-I and RS-II cohorts; 8638 persons) heterozygous female carriers of the minor (A) allele had a significantly increased risk of fracture as compared with the risk among noncarriers of the A allele. The odds ratio in the RS-I cohort was 1.74 (95% confidence interval [CI], 1.19 to 2.55; P = 0.004), and the odds ratio in the RS-II cohort was 2.99 (95% CI, 1.44 to 6.20; P = 0.003). In a combined analysis of the RS-I and RS-II cohorts in a fixed-effect model, the odds ratio was 1.95 (95% CI, 1.39 to 2.74; P<0.001)

(Table 2). We observed no statistical indication of sex-specific effects (P>0.05 for heterogeneity), although associations between carrier status and fracture risk among men in the RS-I cohort were not significant and no fractures were observed in the very small number of male A-allele carriers in the RS-II cohort, which had a shorter follow-up.

Analyses of individual study data for an association with BMD did not show consistent effects. Combined analyses of BMD in the three cohorts showed a small but significantly decreased BMD at the lumbar spine and femoral neck in heterozygous women (P=0.008 and P=0.04, respectively), whereas no significant difference was observed in men (Table 3), again without statistical evidence of heterogeneity between sexes. Correction for BMD in the fracture analysis restricted to the group with BMD and fracture information resulted in a minor decrease in the fracture risk among women.

Functional studies

Electrophoresis of Type I Collagen

No decreased production or overmodification of type I collagen was observed.

Western Blot Analysis

No PLS3 was detected on Western blots in the fibroblast lysates from Patients 1.III-2, 1.IV-2, 1.IV-7, and 1.IV-8, who had the c.235delT variant (Fig. S5 in the Supplementary Appendix). PLS3 production in Patient 3.II-1, who had the c.748+1G→A variant, was decreased. In Patient 4.II-1, who had the c.759_760insAAT variant, and in the index patients from Families 7 and 9 who had the c.321T→A variant, the production of PLS3 was similar to that in controls.

Table 3. BMD at the Femoral Neck and Lumbar Spine with Adjustment for Age and Body-Mass Index, According to Sex, Rotterdam Study Cohort, and rs140121121 Genotype.*

Cohort	Women							Men				
	Genotype 0		Genotype 1		Genotype 2		P Value	Genotype 0		Genotype 2		P Value
	no. of persons	BMD	no. of persons	BMD	no. of persons	BMD		no. of persons	BMD	no. of persons	BMD	
		g/cm²		*g/cm²*		*g/cm²*			*g/cm²*		*g/cm²*	
Femoral neck												
RS-I	2959	0.83±0.002	93	0.81±0.012	—	—	0.14	2165	0.92±0.003	49	0.90±0.018	0.39
RS-II	992	0.89±0.004	31	0.87±0.023	1	1.04±0.126	0.38	851	0.97±0.004	20	1.02±0.028	0.09
RS-III	1113	0.92±0.004	34	0.89±0.021	—	—	0.13	860	0.99±0.004	09	0.98±0.043	0.92
All cohorts	5064	0.85±0.001	158	0.84±0.009	—	—	0.04	3876	0.95±0.002	78	0.94±0.014	0.40
Lumbar spine												
RS-I	2970	1.04±0.003	90	1.03±0.018	—	—	0.86	2176	1.16±0.004	49	1.12±0.027	0.12
RS-II	1004	1.11±0.006	31	1.03±0.032	1	1.33±0.180	0.02	853	1.21±0.006	20	1.33±0.040	0.002
RS-III	1017	1.18±0.005	32	1.13±0.030	—	—	0.09	754	1.25±0.007	09	1.26±0.061	0.89
All cohorts	4991	1.08±0.002	153	1.05±0.014	—		0.008	3783	1.19±0.003	78	1.19±0.021	0.81

* Plus–minus values are means ±SE. Genotype 0 was defined as T in men and TT in women, genotype 1 as TA in women, and genotype 2 as A in men and AA in women.

Immunofluorescence Microscopy

Staining with rhodamine phalloidin (Fig. S6 in the Supplementary Appendix) visualizes stress fibers, a specific type of contractile F-actin bundles. An investigator who was unaware of the clinical and molecular genetic data observed no clear differences in the quantity or quality of stress fibers in patients with the pathogenic c.235delT *PLS3* variant, as compared with controls.

Mechanosensitivity Studies

All cell lines produced a small amount of nitric oxide in response to fluid shear stress. Statistical analysis with the use of the Mann–Whitney U test showed no significant differences among controls, patients with osteogenesis imperfecta, and patients with pathogenic *PLS3* variants.

In Vivo Characterization of pls3 Knockdown in Zebrafish

Zebrafish with *pls3* knockdown had severe dysplasia of craniofacial skeletal elements (Fig. S7A and S7B, Fig. S8A and S8B, and Fig. S9C in the Supplementary Appendix). Gross morphologic abnormalities were observed in the knockdown zebrafish larvae, which were specific and could be reversed dose-dependently by injection of human *PLS3* mRNA (Fig. S7C and S7D, Fig. S8C and S8D, Fig. S9A and S9B, and Fig. S10 in the Supplementary Appendix). Furthermore, the muscle tissue in the knockdown larvae, characterized by a predominance of F-actin, was also deformed (Fig. S8A and S8B in the Supplementary Appendix). Immunohistochemical colocalization experiments revealed a distinct actin-bundling function of pls3 in the developing bone structure (Fig. S11 in the Supplementary Appendix).

Discussion

We identified five pathogenic variants in *PLS3* in Families 1 through 5, with osteoporosis and osteoporotic fractures manifested in childhood in the majority of hemizygous male family members. The clinical picture in heterozygous women from Families 1 and 2 ranged from normal bone density and an absence of fractures to early-onset osteoporosis. Factors such as differences in overall and local X-chromosome inactivation, postmenopausal status, and immobility could play a role.

In addition, we identified a rare variant in *PLS3*, c.321T→A in exon 4 (SNP rs140121121) in Families 6 through 10. The prevalence of this variant was significantly increased in our group of 95 male patients without *COL1A1* or *COL1A2* mutations who had been referred for diagnosis or ruling out of osteogenesis imperfecta type I. The clinical symptoms of patients in Families 6 through 10 were generally less severe and had a later onset (absent in one case) than those in Families 1 through 5 with loss-of-function variants in *PLS3*. We hypothesized that the rs140121121 SNP may be associated with fractures, decreased BMD, or both in the general population.

A combined analysis of two cohorts (RS-I and RS-II) of 8638 elderly Dutch persons showed that heterozygous women had an increased odds of fracture of 1.95 (95% CI, 1.39 to 2.74) and that the SNP was significantly associated with decreased BMD. However, the association with fracture risk was not fully explained by BMD, which suggests that other factors leading to decreased bone strength may be involved. Associations in hemizygous men were not significant, a finding that may be due to the small size of this group or may indicate that additional (possibly genetic) factors play a role. The associations of the SNP with fractures and BMD in the general population need to be replicated in larger cohorts worldwide.

Our findings indicate that PLS3 has bone-regulatory properties. Overexpression

of PLS3 has been reported to act as a protective modifier of spinal muscular atrophy, facilitating axonal growth and presynaptic F-actin-dependent processes at the neuromuscular junction.[17,18] A knockdown of *pls3* in zebrafish was used in an investigation of motor axon development.[17] Since no other animal models were available, we used this model[17] to analyze the role of PLS3 in skeletal development. Malformations of developing craniofacial bone structure, body axis, and tail were present and could be reversed dose-dependently by the administration of human *PLS3* mRNA. Muscles that contained F-actin appeared to be deformed as well, which is notable because the formation of pharyngeal cartilage and the formation of muscle occur simultaneously.[19] Immunohistochemical colocalization experiments confirmed a distinct actin-bundling function of pls3 in developing bone structure. Taken together, the in vivo data suggest that PLS3 may be a regulator of bone development.

The exact mechanism by which *PLS3* mutations cause osteoporosis and fractures is unknown. Fimbrin, the chicken homologue of PLS3,[20] is abundant in osteocyte dendrites.[21-23] These dendrites are important for mechanosensing (converting mechanical signals into intracellular biochemical signals to osteoblasts and osteoclasts).[24] The loss of sensor-cell mechanosensitivity has been proposed as a cause of osteoporosis.[25] We hypothesize that *PLS3* mutations lead to decreased mechanosensing of osteocytes, with subsequent dysregulation of bone modeling or remodeling, which results in osteoporosis and fractures. Bone tissue from patients with *PLS3* mutations will be needed for investigation of mechanosensing in osteocytes.

In conclusion, we identified loss-of-function variants in *PLS3* as a monogenetic cause of X-linked osteoporosis and osteoporotic fractures. We propose diagnostic analysis of *PLS3* in boys and men who have clinical or radiologic signs of an inherited bone disorder with low BMD and fractures, early-onset osteoporosis, or a presumptive diagnosis of osteogenesis imperfecta type I without *COL1A1* or *COL1A2* mutations. Among elderly study participants, we identified a rare *PLS3* variant, which was associated with decreased BMD and a risk of fracture among

heterozygous women that was two times as high as that among noncarriers, indicating genetic variation in *PLS3* as a novel factor involved in common, multifactorial osteoporosis.

Supported by grants to the Rotterdam Study from Erasmus Medical Center and Erasmus University, Rotterdam; the Netherlands Organization of Scientific Research (NWO) Investments (175.010.2005.011 and 911-03-012); the Research Institute for Diseases in the Elderly (014-93-015 and RIDE2); the Ministry of Education, Culture, and Science; the Ministry for Health, Welfare, and Sports; Directorate-General XII of the European Commission; the Netherlands Genomics Initiative and NWO Project (050-060-810); the Netherlands Consortium of Healthy Aging; and the Municipality of Rotterdam. The zebrafish studies were supported by grants from the European Union Program FP7 Program (2012 305121), Deutsche Forschungsgemeinschaft (Wi945-14/1, to Dr. Wirth), and SMA-Europe (to Dr. Riessland). The *col1a1:eGFP* transgenic line was a gift from Dr. Shannon Fisher to Dr. Hammerschmidt's laboratory.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank the participants and the research center staff in the Rotterdam study; the contributors of the fracture data set, Mr. Pascal Arp for genotyping rs140121121, Dr. Fernando Rivadeneira for advice and help with the statistical analyses in the Rotterdam study, Mr. Seyyed Mohsen Hosseini-Barkooie for generating the PLS3- RNA expression vector, Dr. Birgit de Witte for help with the statistical analyses of the mechanosensitivity experiments, Dr. Jan M. Cobben for assistance with the linkage analysis, and Dr. René J.P. Musters for advice on the immunofluorescence experiments.

References

1. **Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N**. The diagnosis of osteoporosis. *J Bone Miner Res* 1994; 9:1137-41.

2. **Laine CM, Koltin D, Susic M, et al**. Primary osteoporosis without features of OI in children and adolescents: clinical and genetic characteristics. *Am J Med Genet A* 2012;158A:1252-61.

3. **Hartikka H, Mäkitie O, Männikkö M, et al**. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. *J Bone Miner Res* 2005;20:783-9.

4. **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 Genet* 2012;44:491-501.

5. **Laine CM, Joeng KS, Campeau PM, et al.** WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *N Engl J Med* 2013;368:1809-16.

6. **Keupp K, Beleggia F, Kayserili H, et al**. Mutations in WNT1 cause different forms of bone fragility. *Am J Hum Genet* 2013; 92:565-74.

7. **van Dijk FS, Dalgleish R, Malfait F, et al**. Clinical utility gene card for: osteogenesis imperfecta. *Eur J Hum Genet* 2013;21: 698-9.

8. **Sillence DO**. Bone dysplasia: genetic and ultrastructural aspects with special reference to osteogenesis imperfecta. *Ann Arbor*, MI: University Microfilms, 1980.

9. **Ameziane N, Sie D, Dentro S, et al**. Diagnosis of Fanconi anemia: mutation analysis by next-generation sequencing. *Anemia* 2012;2012:132856.

10. **Clarke L, Zheng-Bradley X, Smith R, et al**. The 1000 Genomes Project: data management and community access. *Nat Methods* 2012;9:459-62.

11. **Hofman A, van Duijn CM, Franco OH, et al**. The Rotterdam Study: 2012 objectives and design update. *Eur J Epidemiol* 2011;26:657-86.

12. **Delanote V, Vandekerckhove J, Gettemans J**. Plastins: versatile modulators of actin organization in (patho)physiological cellular processes. *Acta Pharmacol Sin* 2005;26:769-79.

13. **Bacabac RG, Smit TH, Van Loon JJWA, Doulabi BZ, Helder M, Klein-Nulend J**. Bone cell responses to high-frequency vibration stress: does the nucleus oscillate within the cytoplasm? *FASEB J* 2006;20: 858-64.

14. **van der Pauw MTM, Klein-Nulend J, van den Bos T, Burger EH, Everts V, Beertsen W**. Response of periodontal ligament fibroblasts and gingival fibroblasts to pulsating fluid flow: nitric oxide and prostaglandin E2 release and expression of tissue non-specific alkaline phosphatase activity. *J Periodontal Res* 2000;35:335-43.

15. **Kague E, Gallagher M, Burke S, Parsons M, Franz-Odendaal T, Fisher S**. Skeletogenic fate of zebrafish cranial and trunk neural crest. *PLoS One* 2012;7(11): e47394.

16. **Clayton D**. Testing for association on the X chromosome. *Biostatistics* 2008;9: 593-600.

17. **Oprea GE, Kröber S, McWhorter ML, et al**. Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 2008;320:524-7.

18. **Ackermann B, Kröber S, Torres-Benito L, et al**. Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 2013;22:1328-47.

19. **Shwartz Y, Farkas Z, Stern T, Aszódi A, Zelzer E**. Muscle contraction controls skeletal morphogenesis through regulation of chondrocyte convergent extension. *Dev Biol* 2012;370:154-63.

20. **de Arruda MV, Watson S, Lin CS, Leavitt J, Matsudaira P**. Fimbrin is a homologue of the cytoplasmic phosphoprotein plastin and has domains homologous with calmodulin and actin gelation proteins. *J Cell Biol* 1990;111:1069-79.

21. **Bonewald LF**. The amazing osteocyte. *J Bone Miner Res* 2011;26:229-38.

22. **Tanaka-Kamioka K, Kamioka H, Ris H, Lim SS**. Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections. *J Bone Miner Res* 1998;13:1555-68.

23. **Kamioka H, Sugawara Y, Honjo T, Yamashiro T, Takano-Yamamoto T**. Terminal differentiation of osteoblasts to osteocytes is accompanied by dramatic changes in the distribution of actin-binding proteins. *J Bone Miner Res* 2004;19:471-8.

24. **Weinbaum S, Duan Y, Thi MM, You L**. An integrative review of mechanotransduction in endothelial, epithelial (renal) and dendritic cells (osteocytes). *Cell Mol Bioeng* 2011;4:510-37.

25. **Mulvihill BM, Prendergast PJ.** Mechanobiological regulation of the remodelling cycle in trabecular bone and possible biomechanical pathways for osteoporosis. *Clin Biomech* (Bristol, Avon) 2010;25:491-8.

10

Osteoporotic Vertebral Fractures During Pregnancy: Be Aware of a Potential Underlying Genetic Cause

Osteoporotic Vertebral Fractures During Pregnancy: Be Aware of a Potential Underlying Genetic Cause

Authors:

Natalia Campos-Obando, Ling Oei, Lies H. Hoefsloot, Rosalie M. Kiewiet, Caroline C. W. Klaver, Marleen E. H. Simon, and M. Carola Zillikens

Status:

Published in *J Clin Endocrinol Metad* 2014;99:1107-11

Context: Although the baby growing in its mother's womb needs calcium for skeletal development, osteoporosis and fractures very rarely occur during pregnancy.

Case Presentation: A 27-year-old woman in the seventh month of her first pregnancy contracted midthoracic back pain after lifting an object. The pain was attributed to her pregnancy, but it remained postpartum. Her past medical history was uneventful, except for severely reduced vision of her left eye since birth. Family history revealed that her maternal grandmother had postmenopausal osteoporosis and her half-brother had three fractures during childhood after minor trauma. Her height was 1.58 m; she had no blue sclerae or joint hyperlaxity. Laboratory examination including serum calcium, phosphate, alkaline phosphatase, creatinine, β-carboxyterminal cross-linking telopeptide of type I collagen, 25-hydroxyvitamin D, and TSH was normal. Multiple thoracic vertebral fractures were diagnosed on x-ray examination, and dual-energy x-ray absorptiometry scanning showed severe osteoporosis (Z-scores: L2–L4, -5.6 SD; femur neck, -3.9 SD). DNA analyses revealed two compound heterozygous missense mutations in *LRP5*. The patient's mother carried one of the *LRP5* mutations and was diagnosed with osteoporosis. Her half-brother, treated with cabergoline for a microprolactinoma, also had osteoporosis of the lumbar spine on dual-energy x-ray absorptiometry and carried the same *LRP5* mutation. The patient was treated with risedronate for 2.5 years. Bone mineral density and back pain improved. She stopped bisphosphonate use 6 months before planning a second pregnancy.

Conclusion: Our patient was diagnosed with osteoporosis pseudoglioma syndrome/familial exudative vitreoretinopathy. Potential underlying genetic causes should be considered in pregnancy-associated osteoporosis with implications for patients and relatives. More studies regarding osteoporosis treatment preceding conception are desirable.

Pregnancy- and lactation-associated osteoporosis (PLO) with the occurrence of fragility fractures mainly of the vertebral bodies was first described as a syndrome by Nordin and Roper (1) in 1955. It is most commonly observed in the third trimester or early postpartum in women presenting with severe and prolonged back pain and sometimes height loss. The prevalence is unknown, and so far about 120 case reports have been reported (2). The etiology is also not known, although a role of calciotropic hormones such as PTHrP has been suggested (3, 4). Most of the cases have been reported in primigravid women (3). There are no guidelines for treatment due to the lack of controlled trials.

Table 1. Laboratory and Imaging Studies

Lab Values	**Reference Values**	**Index Patient, III:2**	**Patient's Mother, II:2**	**Patient's Half-brother, III:3**
Serum				
Calcium, mmol/L	2.25–2.65	2.35	2.26	2.31
Phosphate, mmol/L	0.8 –1.4	1.39	0.96	1.19
Creatinine, µmol/L	55–90	67	66	82
TSH, mU/L	0.4 – 4.3	1.26	4.10	0.55
ALP, U/L	<97	83	76	71
Bone-specific ALP, g/L	<20.1	**22.6**	N/A	**21.8**
25-Hydroxyvitamin D, nmol/L	>50	59	101	46
bCTX, µg/L	<0.56	0.11	N/A	0.88
Urine				
24-h calcium, mmol/24 h	2.5–7.5	**1.9**	7.2	N/A
Urine spot sample, mmol/L				3.55
DXA scan	Cut-off for osteoporosis			
Lumbar spine L2--L4 (T-score)	≤ – 2.5 SD	**– 5.7**	**– 3.2**	– 1.4
Lumbar spine L2--L4 (Z-score)	≤ – 2.0 SD	**– 5.6**	**– 2.8**	**– 2.1**
Femoral neck (T-score)	≤ – 2.5 SD	**– 3.9**	– 0.5	0.0
Femoral neck (Z-score)	≤ – 2.0 SD	**– 3.9**	0.0	– 0.8

Abbreviations: ALP, alkaline phosphatase; bCTX, β-carboxyterminal cross-linking telopeptide of type I collagen; N/A, measurement not available.

Values outside of reference range are marked bold.

Another form of rare pregnancy-associated osteoporosis is called transient osteoporosis of pregnancy. Transient osteoporosis of pregnancy usually presents in the third trimester of pregnancy, sometimes with very severe pain while walking or standing, usually localized in the hip, and sometimes leading to hip fracture (5). Radiographs can show severe localized loss of bone mass, whereas only edema may be visible in magnetic resonance imaging in early stages. This condition usually fades within a few months after delivery. Additionally, pregnancy and lactation might lead to bone loss in patients with pre-existent osteoporosis attributable to genetic causes of low bone mineral density (BMD). As a consequence, these patients may become clinically manifest and develop fractures during this period. In this case report, we describe the clinical picture of a 27-year-old woman diagnosed with vertebral fractures and osteoporosis shortly after pregnancy. We will discuss potential causes of pregnancy-associated osteoporosis, its clinical consequences, and issues to take into account concerning patient management.

Case Presentation

A 27-year-old Caucasian woman in the seventh month of her first pregnancy complained of midthoracic back pain after bending over to lift a nonheavy object. The pain remained with differing intensity and was attributed to her pregnancy. After the delivery of a healthy child, the back pain prevented her from lifting her baby. She breastfed her baby for about 4 weeks. Because physical therapy had no effect on the pain, she was referred to an internist about 3 months after delivery. Her past medical history was uneventful without fractures, but she reported a severely reduced vision of her left eye since birth of unknown etiology, treated unsuccessfully with patches on the right eye. She consumed two to three dairy products daily. There was no history of abnormal menstrual cycle, smoking, alcohol, or medication use (such as corticosteroids) except for over-the-counter calcium and vitamin D supplements. Family history revealed that her maternal grandmother had postmenopausal osteoporosis, her grandfather had ankylosing spondylitis, and her only sibling (a half-brother) had experienced three fractures

during childhood after minor trauma. On physical examination, her height was 1.58 m (5 ft 2 in), her weight was 53 kg (117 lb), and she had no blue sclerae and no joint or skin hyperlaxity. Her maximally corrected visual acuity was 0.16 + + left (S-6.50 = C-2.75 X 22) and 1.0– right (S-5.75 = C-1.25 X 170). Further ophthalmological examination revealed amblyopia in the left eye and changes compatible with a mild form of familial exudative vitreoretinopathy (FEVR) in both eyes. There was normal form and function of the spinal column, which was slightly painful during flexion and extension. Except for an increase in bone-specific alkaline phosphatase and low urinary calcium excretion, there were no abnormalities on laboratory examination (Table 1). Spinal x-ray showed end-plate compressions of thoracic vertebrae (T7, -9, -10, and -12; Figure 1). Dual-energy x-ray absorptiometry (DXA) scanning performed approximately 3 months after delivery showed severe osteoporosis (Z-scores: L2–L4, –5.6 SD; femur neck, –3.9 SD) (Table 1). A biopsy of the iliac crest revealed coarse trabeculae with loss of connectivity and a strongly increased bone turnover, but no evidence for mastocytosis or osteomalacia. After obtaining informed consent, DNA analysis was performed and showed no mutations in the *COL1A1* or *COL1A2* genes, only a polymorphism in the *COL1A1* gene that has been reported in juvenile osteoporosis but also in nonaffected family members.

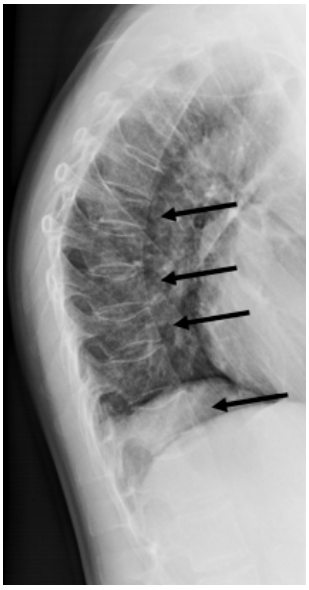


Figure 1. Lateral spinal x-ray image of the index patient. The black arrows show end-plate compressions of thoracic vertebrae at T7, T9, T10, and T12.

This makes a mild form of osteogenesis imperfecta unlikely. It is important, however, to notice that 10% of patients with clinical osteogenesis imperfecta have no detectable mutations in the exons for *COL1A1* and *COL1A2* (6). DNA analyses of the *LRP5* gene revealed two compound heterozygous mutations, c.1519G>A (p.Gly507Ser) and c.3758G>T (p.Cys1253Phe). Subsequently, family screening with DXA and DNA analyses were performed (Figure 2). The mother of the patient, recently postmenopausal, is a carrier of the *LRP5* c.3758G>T mutation and was diagnosed with osteoporosis on a DXA scan (Z-scores: lumbar spine, –2.8 SD; femoral neck, +0.0 SD). Spine radiography showed mild anterior wedging (less than 25%) of three thoracic vertebrae. The patient's half-brother, treated with cabergoline for a microprolactinoma, carried the same *LRP5* c.3758G>T mutation. He also had osteoporosis on the DXA scan (Z-scores: lumbar spine, -2.1 SD; femoral neck, +0.0 SD) and had sustained three fractures after minimal trauma at a young age, as described before. He had no vertebral fractures on spine radiography. The mother and half-brother had no v isual impairments. The father of the patient was deceased and could therefore not be tested. Surprisingly, the c.3758G>T mutation was not detected in DNA from the maternal grandmother with osteoporosis. This indicates that the mutation was inherited from the maternal grandfather or a de novo mutation and that the grandmother may have had common osteoporosis. The patient was treated with risedronate for 2.5 years. BMD and back pain improved. She stopped the use of bisphosphonate 6 months before planning a second pregnancy.

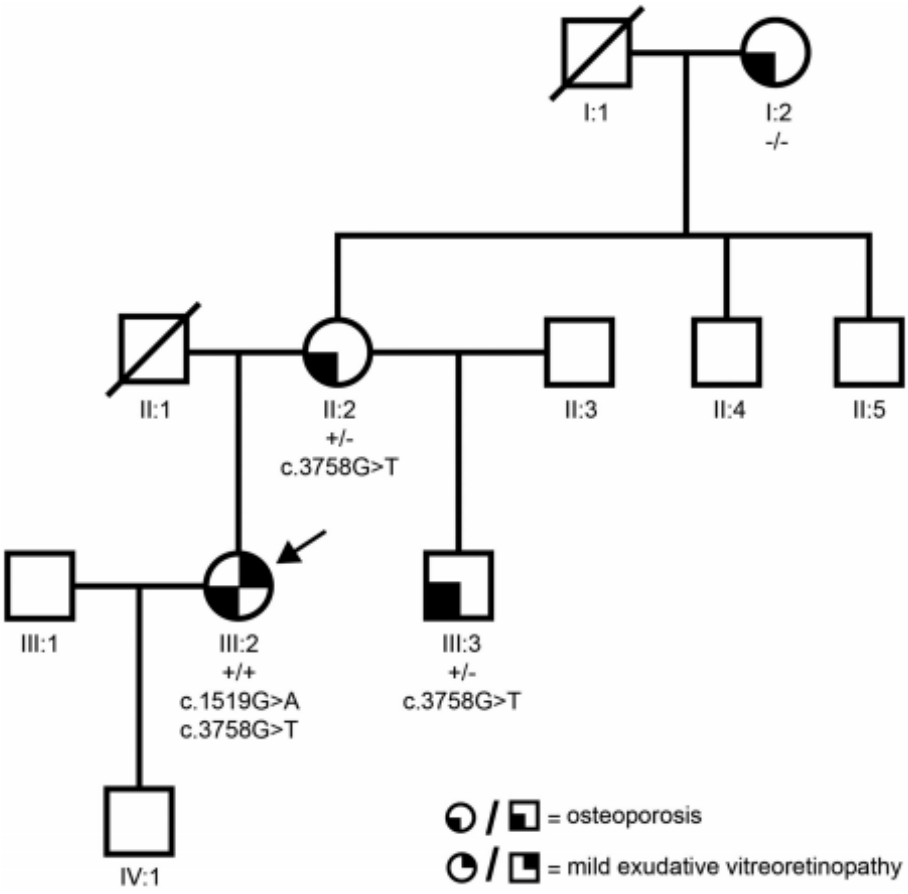


Figure 2. Pedigree and genotypes of the family. Index patient (III:2) is indicated with an arrow. She is compound heterozygous for the c.1519G>A and the c.3758G>T mutations. Subjects II:2 and III:3 are carriers of the c. 3758G>T mutation. Black squares within cicles and square represent low BMD (below left) and mild exudative vitreoretinopathy (above right).

Discussion

In this case report we describe the clinical picture of a young woman without a history of fractures. She presented in the third trimester of her first pregnancy with disabling back pain that persisted after delivery and was caused by fractures of multiple thoracic vertebrae. She had a severely reduced BMD on DXA scanning. We considered the diagnosis of PLO, but we identified a genetic cause underlying her condition. PLO is a rare heterogeneous disorder of unknown etiology. It is characterized by the occurrence of fragility fractures mostly in the spine and severe back pain presenting typically in the third trimester of gestation or early postpartum period (3). In PLO, whereas some patients improve spontaneously after giving birth or stopping lactation, others need medical treatment and continue to have decreased BMD (7). Pre-existing secondary causes of osteoporosis, such as vitamin D deficiency, celiac disease, anorexia nervosa, mastocytosis, and hyper(para)thyroidism, should always be ruled out. Pregnancy and lactation may lead to up to 5–10% loss of mainly trabecular bone, especially during breast-feeding. However, almost complete recovery occurs in most cases within 6 to 12 months (8) and thus cannot explain the verylow BMD in our patient unless BMD was already compromised before pregnancy due to other reasons. In patients with PLO, a high prevalence of fractures has been reported in their mothers (9) and of osteopenia in their offspring (10), leading to the suggestion of an underlying (genetically determined) low peak bone mass (8, 9).

In our patient, we suspected an underlying monogenetic bone disease due to the severity of her osteoporosis. Analysis of her half-brother and mother confirmed a familial component. The history of severely reduced vision in one eye since birth led to suspicion of osteoporosis pseudoglioma (OPPG) syndrome, an autosomal recessive disorder characterized by early onset osteoporosis and blindness (OMIM no. 259770). OPPG is a rare disease with an estimated incidence of 1/2 000 000 and a carrier frequency of 1/700 (11), caused by biallelic loss of function mutations in *LRP5* (12). LRP5 (low-density lipoprotein receptor-related protein 5) is a cell-surface protein receptor that plays a key role in several intracellular

signaling pathways, mainly Wnt and Norrin signaling (12). Mutations in *LRP5* are also involved in FEVR (13) (FEVR/ exudative vitreoretinopathy 4, OMIM no. 133780), a hereditary blinding disorder with a highly variable phenotype even within the same family (14). Both autosomal recessive and autosomal dominant inheritance can occur. FEVR caused by *LRP5* mutations is associated with low bone mass, in contrast to FEVR caused by mutations in other genes (eg, *FZD4* or *NDP*) (14). OPPG and FEVR caused by *LRP5* mutations are therefore disorders with an overlapping phenotype. It has been suggested by Qin et al (14) that OPPG and FEVR caused by mutations in *LRP5* are part of a single phenotypic spectrum with both ocular and bone manifestations. DNA analysis in our patient showed compound heterozygosity for two missense mutations in the LRP5 gene. The c.1519G>A (p.Gly507Ser) mutation is predicted to induce a minor chemical change of an evolutionary strongly conserved amino acid with introduction of an alternate splice acceptor site, and when present in homozygous state induces OPPG with very low BMD levels (15). On the other hand, c.3758G>T (p.Cys1253Phe) is predicted to induce a major chemical change of an evolutionary strongly conserved amino acid and has been previously described in recessive FEVR (13). Because most patients with OPPG are congenitally blind or become blind by the age of 25 years (11, 15–17), it is remarkable that our patient had relatively mild signs of exudative vitreoretinopathy, and a diagnosis of recessive FEVR might be considered as well (18), although osteoporosis is usually less severe than in OPPG (11, 14, 15). The mother and half-brother carrying the *LRP5* c.3758G>T mutation that has been previously described in recessive FEVR also had decreased BMD. Although OPPG follows an autosomal recessive pattern of inheritance, heterozygous carriers can exhibit mildly reduced BMD (19).

Heterozygous mutations in *LRP5* are associated with primary osteoporosis in children (20). Moreover, in genome-wide meta-analyses the *LRP5* locus was significantly associated with BMD and fracture risk (21), broadening the spectrum of bone abnormalities related to genetic variation in *LRP5*.

We treated our patient with risedronate after she told us she did not want to get

pregnant for at least 2 years, and she continued the use of oral contraceptives. Bisphosphonates are contraindicated in pregnancy. Animal studies with high doses have shown maternal and fetal toxicity, and there is concern of treating premenopausal women with these drugs because they are retained in bone for several years (22). A recent study of the literature that identified 78 cases of pregnancies involving exposure to bisphosphonates before conception or during pregnancy did not demonstrate serious adverse effects. Despite this, cases of increased spontaneous abortions, shortened gestational age, low neonatal birth weight, and transient hypocalcemia of the newborn were reported (23). Although bisphosphonates share the same core structure, their binding affinity to hydroxyapatite crystals varies among them; those with higher affinity display longer skeletal retention. It has been found that the ranking order for hydroxyapatite affinity from highest to lowest is zoledronate > alendronate > ibandronate = risedronate > etidronate (24). We chose a bisphosphonate with relatively low skeletal retention. We advised the patient to stop treatment at least 6 months before stopping birth control because risedronate levels have not been detected in urine 5 months after cessation of therapy (25). We would nevertheless advise close monitoring of pregnancy and intrauterine growth, check for neonatal hypocalcemia, and report on outcome. Also, we advised our patient to limit or avoid lactation after a subsequent pregnancy to prevent further maternal bone loss associated with breast-feeding (8). Alternatively, newer medications without long-term bone retention could be considered as off-label treatment in premenopausal women at very high risk for fractures who wish to become pregnant. However, in theory, stopping these drugs before becoming pregnant could lead to increased bone loss during pregnancy.

Conclusion

We report the clinical picture of a 27-year-old woman who suffered from disabling back pain during pregnancy and was diagnosed with multiple vertebral fractures and severe osteoporosis after delivery. We made the diagnosis of severe osteoporosis due to compound heterozygous mutations in the *LRP5* gene with mild

exudative vitreoretinopathy as part of a spectrum of diseases named "osteoporosis pseudoglioma syndrome" and "familial exudative vitreoretinopathy." Thus, our patient was genetically predisposed, and pregnancy further exacerbated her osteoporosis, resulting in vertebral fractures. We propose screening for an underlying monogenetic bone disorder in patients with PLO and one of the following features: a severely reduced BMD (Z-scores < –2.0 SD); a family history of osteoporosis or fragility fractures, joint hypermobility, blue sclerae, congenital blindness, or severely reduced vision; or a history of fractures before pregnancy (eg, testing for mutations in *collagen 1A1* and *1A2* genes, *LRP5*, *WNT1* [26], and *LGR4* [27], and for the recently reported *PLS3* gene [28]). A genetic diagnosis has implications for the patient and relatives. More studies regarding bisphosphonate treatment and newer osteoporosis drugs preceding conception are desirable.

Acknowledgments

Address all correspondence and requests for reprints to: M. Carola Zillikens, MD, PhD, PO Box 2040, 3000 CA Rotterdam, The Netherlands.
E-mail: m.c.zillikens@erasmusmc.nl. Disclosure Summary: All authors have no conflicts of interest.

References

1. **Nordin BE, Roper A**. Post-pregnancy osteoporosis; a syndrome? *Lancet* 1955;268:431– 434.

2. **Choe EY, Song JE, Park KH, et al**. Effect of teriparatide on pregnancy and lactation-associated osteoporosis with multiple vertebral fractures. *J Bone Miner Metab* 2012;30:596 – 601.

3. **O'Sullivan SM, Grey AB, Singh R, Reid IR**. Bisphosphonates in pregnancy and lactation-associated osteoporosis. *Osteoporos Int* 2006;17:1008 –1012.

4. **Sowers MF, Hollis BW, Shapiro B, et al**. Elevated parathyroid horm ne-related peptide associated with lactation and bone density loss. *JAMA* 1996;276:549 –554.

5. **Maliha G, Morgan J, Vrahas M**. Transient osteoporosis of pregnancy. *Injury* 2012;43:1237–1241.

6. **van Dijk FS, Dalgleish R, Malfait F, et al**. Clinical utility gene card for: osteogenesis imperfecta. *Eur J Hum Genet* doi:10.1038/ejhg.2012.210.

7. **Lampropoulou-Adamidou K, Trovas G, Stathopoulos IP, Papaioannou NA**. Case report: teriparatide treatment in a case of severe pregnancy -and lactation-associated osteoporosis. *Hormones (Athens)* 2012;11:495–500.

8. **Kovacs CS**. Calcium and bone metabolism disorders during pregnancy and lactation. *Endocrinol Metab Clin North Am* 2011;40:795– 826.

9. **Dunne F, Walters B, Marshall T, Heath DA**. Pregnancy associated osteoporosis. *Clin Endocrinol* (Oxf) 1993;39:487– 490.

10. **Carbone LD, Palmieri GM, Graves SC, Smull K**. Osteoporosis of pregnancy: long-term follow-up of patients and their offspring. *Obstet Gynecol* 1995;86:664 – 666.

11. **Ai M, Heeger S, Bartels CF, Schelling DK**. Clinical and molecular findings in osteoporosis-pseudoglioma syndrome. *Am J Hum Genet* 2005;77:741–753.

12. **Gong Y, Slee RB, Fukai N, et al**. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 2001;107:513–523.

13. **Nikopoulos K, Venselaar H, Collin RW, et al**. Overview of the mutation

spectrum in familial exudative vitreoretinopathy and Norrie disease with identification of 21 novel variants in FZD4, LRP5, and NDP. *Hum Mutat* 2010;31:656 – 666.

14. **Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H**. Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations in the LRP5 and/or FZD4 genes. *Hum Mutat* 2005;26:104 –112.

15. **Tüysüz B, Bursalı A, Alp Z, Suyugül N, Laine CM, Mäkitie O**. Osteoporosis-pseudoglioma syndrome: three novel mutations in the LRP5 gene and response to bisphosphonate treatment. *Horm Res Paediatr* 2012;77:115–120.

16. **Gong Y, Vikkula M, Boon L, et al**. Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chrom some region 11q12–13. *Am J Hum Genet* 1996;59:146 –151.

17. **Somer H, Palotie A, Somer M, Hoikka V, Peltonen L**. Osteoporosispseudoglioma syndrome: clinical, morphological, and biochemical studies. *J Med Genet* 1988;25:543–549.

18. **Downey LM, Bottomley HM, Sheridan E, et al**. Reduced bone mineral density and hyaloid vasculature remnants in a consanguineous recessive FEVR family with a mutation in LRP5. *Br J Ophthalmol* 2006;90:1163–1167.

19. **Laine CM, Chung BD, Susic M, et al**. Novel mutations affecting LRP5 splicing in patients with osteoporosis-pseudoglioma syndrome (OPPG). *Eur J Hum Genet* 2011;19:875– 881.

20. **Hartikka H, Mäkitie O, Männikkö M, et al**. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. *J Bone Miner Res* 2005;20:783–789.

21. **Estrada K, Styrkarsdottir U, Evangelou E, et al.** Genome-wide metaanalysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet* 2012;44:491–501.

22. **Chan B, Zacharin M**. Maternal and infant outcome after pamidronate treatment of polyostotic fibrous dysplasia and osteogenesis imperfecta before conception: a report of four cases. *J Clin Endocrinol Metab* 2006;91:2017–2020.

23. **Stathopoulos IP, Liakou CG, Katsalira A, et al**. The use of bisphospho-

nates in women prior to or during pregnancy and lactation. *Hormones* (*Athens*) 2011;10:280 –291.

24. **Nancollas GH, Tang R, Phipps RJ, et al**. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. *Bone* 2006;38:617– 627.

25. **Peris P, Torra M, Olivares V, et al.** Prolonged bisphosphonate release after treatment in women with osteoporosis. Relationship with bone turnover. *Bone* 2011;49:706 –709.

26. **Laine CM, Joeng KS, Campeau PM, et al**. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *N Engl J Med* 2013;368:1809 –1816.

27. **Styrkarsdottir U, Thorleifsson G, Sulem P, et al**. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. *Nature* 2013;497:517–520.

28. **van Dijk FS, Zillikens MC, Micha D, et al**. PLS3 mutations in Xlinked osteoporosis with fractures. *N Engl J Med* 2013;369:1529 – 1536

11

Genome-Wide Association Study of human serum phosphate levels: a large Biobanks approach

Genome-Wide Association Study of human serum phosphate levels: a large Biobanks approach

Authors:

Campos-Obando N, Luan J, Bosman A, Medina-Gómez MC, Rivadeneira F, Uitterlinden AG, Perry JRB, Zillikens MC

Status:

Manuscript in preparation.

Abstract

Phosphate is an abundant anion that exerts key physiological roles in vertebrates, and increased or decreased levels can have health consequences, where increased phosphate levels have been linked to premature ageing and adverse health outcomes in men, but not so much in women. Serum phosphate levels are partially genetically determined with a heritability of ~58% and previous Genome Wide Association Studies (GWAS) in European and Japanese ancestry populations have identified ten loci associated with serum phosphate, explaining ~2% of its variance.

We hereby analyzed 392.655 subjects of European ancestry from the UK Biobank by GWAS, applying stringent significance level and mixed-models control for population stratification and relatedness, and identified 264 independent SNPs determining serum phosphate level at $p < 5x10^{-9}$. These signals were clustered in 182 loci of which 172 are new, and jointly explain 7.62% of serum phosphate variance. We replicated all ten loci previously identified to be associated with serum phosphate levels. These results changed little when interrogating a smaller subset of the UK Biobank (n:354.798) composed of restricted White British ancestry participants. The results from a fine-mapping approach within the main loci confirmed the role of *FGF23* and *ALPL* in P levels but also suggested a key role for other genes not known to be related to phosphate homeostasis. The top hit (rs9469580, $p=1.6 \times 10^{-468}$) maps to the region flanking the Major Histocompatibility Complex (MHC, 6p21.31), whose closest annotated gene (*IP6K3*) encodes for an inositol phosphate kinase but it is not known to exert a role in phosphate homeostasis. The second top hit (rs2970818, $p=1.2 \times 10^{-272}$) mapped to 12p13.32, with *FGF23* as the main annotated gene. The third top hit (rs12132412, $p=1.2 \times 10^{-194}$) mapped to 1p36.12, close to *ALPL* locus.

In summary, we identified 172 new loci associated with serum phosphate and now explain >7% of the variance in serum phosphate levels, opening up possibilities in risk stratification and Mendelian Randomization studies. Further detailed analyses are needed to better understand how the locus in the MHC flanking region is determining serum phosphate levels.

Introduction

Phosphate (P) is indispensable for the normal functioning in mammalian organisms. Its roles span from having structural functions in bone and teeth, being a key factor in energetic reactions and being part of the chemical structure of DNA [1], while more recently discovered roles include the regulation of the mineralization process and the induction of osteocyte differentiation [2]. While low serum levels of P result in severe disease phenotypes, such as rickets in children and osteomalacia in adults due to inadequate bone mineralization, increased serum levels of P are also associated with adverse consequences such as excess mortality, partly explained by arterial calcification [3]. In particular, chronic kidney disease (CKD) patients in advanced stages are prone to P retention and hyperphosphatemia and P binders are a cornerstone of regular treatment in advanced CKD stages [4].

Epidemiologic studies have recently associated higher serum P even within the normal range with adverse health outcomes in the general population such as increased fracture risk [5], low bone mineral density at the lumbar spine [5], coronary artery calcification [6] and, especially, increased mortality risk from chronic obstructive pulmonary disease and cardiovascular causes [7-9]. Remarkably, most adverse outcomes have been described only in men [7, 9]. In addition, the current evidence supports that an increasing yet normal P exerts adverse health outcomes even in population free of chronic kidney disease (CKD) [10].

Twin studies have shown that inter-individual differences in serum P concentration in the normal population are partially genetically determined with an estimated heritability of 58% (95% CI: 53-62%) [11]. Rare Mendelian P-wasting disorders in humans, e.g., hypophosphatemic rickets, are caused by mutations in genes within P metabolism [12, 13]. All efforts towards the elucidation of the genetic architecture of P serum levels provide an opportunity to improve the current understanding of both physiological and pathological axes, and also to discover

potential drug-targets suited for future randomized controlled trials.

Previous genome-wide association studies (GWAS) of serum P levels [14, 15] in European (n:16.264 subjects, from 2010) and in Japanese (n:42.793 subjects, from 2018) ancestry populations have identified ten loci: 1p36.12 (rs1697421), 3q21.1 (rs17265703), 5q35.3 (rs35716097), 6p21.31 (rs9469578), 6q23.2 (rs17060705), 6q23.3 (rs947583), 12p13.2 (rs3903005), 12p13.32 (rs2970818), 17q24.2 (rs35186465) and Xp22.11 (rs178710). These ten loci collectively explain ~2% of the variance in serum P levels, leaving still many loci determining serum P levels undiscovered.

We therefore performed a larger GWAS of P serum level data in the UK Biobank, an extensive resource involving ~500.000 participants genotyped with SNP arrays and subsequently imputed to ~93 million genetic variants from dense reference panels [16]. Our purposes were threefold: 1) to discover common and low-frequency variants related to serum P levels [17, 18]; 2) to explore the genetic architecture of serum P levels in humans, including potential sex differences; and 3) to restrict findings to a small set of potentially causative variants through the application of fine-mapping techniques [19-21] and trans-ethnic meta-analysis [22].

Methods

The UK Biobank (UKBB) is a large cohort assembled at the United Kingdom that involves ~502.000 participants aged 40-69 years and recruited from 2006 until 2010. Dense phenotype and genotype data have gradually become available from it for scientific purposes. Genotyping was carried out at the Affymetrix premises in Santa Barbara, CA, USA using two highly customized Affymetrix arrays genotyping chip (UK BiLEVE Axiom Array or UK Biobank Axiom Array), that were subsequently imputed to the Haplotype Reference Consortium reference panel and to a merged UK10K and 1000 Genomes phase 3 reference panel [16], to include close to 93 million imputed variants. In the UK Biobank

a panel of biomarkers measured in serum recently became available, including serum P levels.

SNP selection criteria

The imputed variants in the UK Biobank have been stored in Oxford BGEN v1.2 format, a binary file system that encodes genotype probabilities in smaller file sizes than other formats and that was adopted and optimized for the full release of the UK Biobank [23]. We selected only bi-allelic SNPs with information score (from IMPUTE4 software [24]) above 0.9, which means an effective sample size of 0.9*N [16]. We set a minor allele frequency threshold of 0.005 for downstream analyses (0.5%).

HLA allele imputation within UK Biobank

Due to the large number of genetic associations identified within the Major Histocompatibility Complex [25], the UK Biobank investigators have imputed HLA alleles using a multi-population reference panel. The imputation was conducted at a 4-digit resolution and achieved high accuracy. As a proof of concept, the investigators also performed analyses (within the white British ancestry subset, see below) for diseases with known HLA associations. The results were consistent with previous studies.

Participant selection criteria

The primary analysis of this manuscript includes 392.655 subjects of white European ancestry, as defined by a k-means clustering algorithm applied to the first four PC calculated from genome-wide SNPs in addition to a consistent self-reported ancestry. The secondary analysis included a stricter set of 354.798 participants of white British ancestry, a list provided by UK Biobank investigators and defined as self-identification of belonging to white British ancestry in addition to the implementation of a Bayesian algorithm to detect outliers and which is

based on the first six PC (Supplementary Section of [16]).

We excluded participants with an estimated glomerular filtration rate less than 39 mL/min because the prevalence of CKD-related hyperphosphatemia becomes prominent below this threshold [26].

Association analyses

Serum P levels were regressed on age, age squared, sex and centers for calculating residuals. These were inverse normal transformed for the association analysis. The GWAS regression models included the following as covariates: sex, genotyping chip (UK BiLEVE Axiom Array or UK Biobank Axiom Array), aliquot and the first ten PCs (as provided by UK Biobank). In addition, sex-stratified analyses were performed for both the white European cohort and the white British subset. We included analysis at the X-chromosome, assuming a random X-inactivation pattern.

We implemented a Bayesian mixed-model approoach (BOLT-LMM, see below) for the association analysis, to increase the effective sample size by inclusion of relatives and conditioning on polygenic predictions, an approach that decreases noise in genetic models [17,18].

The program GCTA [27] was used to obtain all independent signals. The significance level was set at $p < 5x10^{-9}$, previously described as an appropriate threshold for testing both common and low-frequency variants [28]. GCTA applies a sliding window for association analysis of 10 Mb of genome sequence. LD score regression [29] was applied to obtain the intercept and attenuation ratio, although it is well acknowledged that both values increase with sample size, especially at the large scale of the UKBB data. LD score regression was used also to estimate heritability; however this estimate is not the traditional SNP-heritability (proportion of variance explained by all genotyped SNPs) but it rather estimates the proportion of variance explained by common SNPs (excluding those

of large effect), which is usually smaller [17]. As a consequence, our heritability estimates are probably underestimated.

Fine-mapping implementation

Taking advantage of our large sample size and the high quality of imputed SNPs [30], we implemented a statistical fine-mapping approach for the first three top hit loci through PAINTOR (Probabilistic Annotation Integrator), a Bayesian approach that incorporates LD patterns, the genetic strength of association and (optionally) genomic annotations for the identification of potential causative variants [21]. For this analysis, we ran PAINTOR without genomic annotations. The software offers three important advantages: a) it requires no prior specification of causality (e.g., it follows an empirical Bayes approach and estimates it from the data); b) its main output is the (posterior) probability of causality for each variant, which in contrast to *p* values, has a straightforward interpretation, can be compared across studies and can be used for prioritization of variants for functional follow-up studies suited to test biological causality; and c) it allows for the possibility of several variants per locus, an important feature as the number of causative variants per region is unknown a priori. From each locus, we selected only genotyped SNPs or SNPs imputed with INFO score >0.9. Besides the Z-scores for each variant, PAINTOR requires an LD matrix per locus as input; the matrices were estimated from data of European participants from the 1000 Genomes Reference Panel (Phase 3). The provided code also aligns the alleles with 1000 Genomes, a critical step in fine-mapping. As advised, we ran all three loci simultaneously, as it increases efficiency.

Rationale for mixed model implementation

Described by Eisenhart [31] and further elaborated by Henderson [32], mixed models were developed to estimate breeding values in animal pedigrees. The mixed model derives from their incorporation of both fixed and random effects. All predictive regression models by definition have increased power to detect new

loci [33]. In this case, the effect of the SNP being tested is set as fixed effect and the additive genetic background effects (modeled in a Genetic Relationship Matrix, GRM) are treated as random effects. By conditioning on polygenic predictions from genome-wide SNPs, mixed models decrease noise in the association tests and offer an important increase in the effective sample size, which has been estimated close to ~700.000 for the UK Biobank [17].

All SNPs included in the GRM must be of high quality. In particular, BOLT-LMM implements a leave-one-out approach, i.e., it excludes from the GRM the whole chromosome where the individual SNP being tested belongs to. This approach is mandatory to avoid an undesirable loss of power by double fitting of the marker, a phenomenon termed proximal contamination [34].

Importantly, both population stratification and relatedness are well described within the UKBB. Relatedness is not negligible, rather it has been found in close to a third of all participants in UKBB. In this context, the implementation of linear mixed models precludes the exclusion of nearly 30% of the total sample size of the full release. In particular, we applied mixed models as implemented in BOLT-LMM, a well-known software to correct for relatedness, stratification and at the same time to increase power [18]. This package offers a Bayesian approach to test for evidence of non-infinitesimal genetic architecture, recently proposed as the most likely architecture for the majority of traits, in contrast to the classic infinitesimal (polygenic) model [35, 36].

It has been previously shown that the contribution of dominant variance is negligible within the UKBB.

Results

In the first phase of this study, we analyzed 392.655 participants (211.798 women) from the UKBB with white European ancestry and identified 264 conditionally independent SNPs associated with phosphate levels at $p < 5x10^{-9}$. Based on the

traditional definition, these markers can be clustered in 182 loci (summary in **Table 1; Figure 1** displays the Manhattan plot). The interrogation of the X-chromosome showed that three X-chromosomal SNPs (clustered in two loci) in men and one SNP in women reached genome–wide significance. Assuming an additive model, all SNPs jointly explained 7.62% of the variance in phosphate levels. (**Supplementary Table 1a, Online Table 1**).

When we restrict the analysis to participants of White British ancestry (n:354.798, list provided by UKBB), we found 243 independent signals that can be clustered in 172 loci, collectively explaining 7.21% of serum phosphate variance. Consistent with findings from the full cohort, the top hit (rs60447213, p=3.9 x 10^{-437}) was found to lie in the MHC region, in 6p21.31 (**Supplementary Table 1b, Online Table 2**).

Genomic control

The value of lambda for genomic control (λ) [37] assessed through different software and subsets of SNPs fluctuated between 1.19, estimated by EasyStrata, to 1.50, estimated by standard LD score regression (**Table 2a, Figure 2**). It has been previously shown that polygenicity and large sample size can inflate λ even in the absence of confounding due to population stratification and relatedness [38] and for that reason we ran standard LD score regression [26], whose intercept in principle is able to discriminate between polygenicity and confounding. Nevertheless, the intercept from the standard LD score regression has also proved to be susceptible to inflation due to high SNP-heritability and large sample size [17]. Therefore, and as previously advised, we relied instead on the attenuation ratio: a calibrated intercept with formula: (LDSC intercept-1) / (mean χ2 -1) from the LD score baseline model, a stratified LD score regression based on 59 genomic annotations that provides better fit than the standard LD regression in large sample size cohorts [39]. Performing this analysis yielded an attenuation ratio of 0.07 (**Table 2b**), consistent with inflation due to the large sample size and not due to confounding [17].

Estimates of total and partitioned heritability

We obtained estimates of narrow-sense heritability from LD score regression that fluctuated in a narrow range between 11 and 13%, according to standard and LD baseline models, respectively. The regression for partitioned heritability for continuous annotations showed enrichment for enhancer and methylation sites. Nevertheless, this analysis systematically excludes all the regions contained within the Major Histocompatibility Complex.

Non-infinitesimal genetic architecture for serum phosphate levels

We tested for evidence within the UKBB for a non-infinitesimal genetic architecture for phosphate levels following an approach described by Loh and co-authors (Supplementary Section of [17]) and computed the mean and median ratio of χ^2 statistics for BOLT-LMM (non-infinitesimal model) and BOLT-LMM-INF (infinitesimal model) in genotyped SNPs with χ^2 more than 40. We found a mean and median of 1.024 and 1.022, respectively, consistent with a modest increase in power for the non-infinitesimal model – meaning that as expected, serum phosphate level is determined by a few thousand loci and does not support the model of all loci being related, each with a tiny effect [40]. Consistently, the results for the infinitesimal model yielded 266 signals that jointly explained 7.58% of serum phosphate level variance, compared to 7.62% for the non-infinitesimal model.

Allele frequency and effect sizes

We identified 32 low frequency alleles of the 264 independent SNPs identified in the white European cohort. Following a previously described approach for selection signatures [41], we plotted the association between SNP effect sizes (betas) and allele frequency for all significant SNPs annotated with ancestral alleles in 1000 Genomes (**Figure 5**). The plot suggests lower frequencies alleles to have higher absolute betas. This finding requires further research to elucidate

whether it corresponds to a signature of negative selection against variants with large effect, as previously suggested for other traits in the UKBB [41].

Sex differences

We assessed potential sex differences in the genetic landscape for P levels through the estimation of a *p* value for the difference in beta's across sexes. When testing all SNPs that passed QC control (and were therefore included in the conditional analyses), the correlation of beta's across sexes was 0.062. In the genome-wide approach without filtering (i.e., genome-wide) and taking relatedness into account, we found three independent SNPs with a suggestive *p* value for sex difference [42], but did not reach the stringent threshold of $5x10^{-9}$. Nevertheless, these SNPs reached a *p* value for sex difference less than $5x10^{-8}$, the suggestive threshold for this analysis in recently published guidelines [42] (**Supplementary Table 2**). **Figure 3** displays the Miami plot for the whole cohort.

The sex-stratified analyses in the white European cohort yielded 91 independent signals in men, which jointly explain 5.85% of the variance in serum phosphate levels, and 125 independent signals in women, which jointly explain 5.62% of variance in serum phosphate levels.

Loci identified

In addition to being able to replicate the ten loci previously associated with phosphate levels [14, 15] (**Table 3**), our analysis in the much larger UKBB GWAS dataset identified 172 new loci determining serum phosphate levels (**Supplementary Table 1a**).

The most significant top hit leads a cluster of seven SNPs which are located in the short arm of chromosome 6, within the flanking region [43] of the Major Histocompatibility Complex (6p: 24-36 Mb). The sentinel SNP of this cluster (6:33715837, rs9469580, $p= 1x10^{-468}$) lies in 6p21.31 (**Figure 4**), its location is

intergenic and the closest annotated gene is *IP6K3*, which encodes a protein of the inositol phosphokinase family whose function is not known to be related to phosphate homeostasis. In total, chromosome 6 harbors at least 34 independent SNPs associated with phosphate levels which could be clustered in 17 loci (**Supplementary Table 1a**). Nine SNPs lay in the region flanking the MHC (**Table 4**); from those SNPs a total of seven independent SNPs clustered in the top hit locus (6p21.31).

The second top hit leads a cluster of 11 SNPs located in the short arm of chromosome 12, within 12p13.32. The sentinel SNP of this cluster (12:4606168, rs297081=1.2x10^{-272}) lies close to *FGF6*, *C12orf4* and *FGF23* as annotated genes. This locus has been previously described, and it is well known that FGF23 encodes a homonymous protein which is a potent phosphaturic agent acting in renal proximal tubules and in collaboration with α-klotho as cofactor.

The third top hit leads a cluster of 14 SNPs which are located in the short arm of chromosome 1 (1p36.12). The sentinel of this cluster (1:21820042; rs12132412, p=1.2 x 10^{-194}) has *ALPL* as main annotated gene (**Figure 4**).

The interrogation of the X-chromosome showed three independent primary signals in men which were clustered in two loci; the sentinel SNP (rs178676, p=1.6 x 10^{-71}) has *PHEX* as the main annotated gene. Women showed one primary signal (rs178688, p=9.6 x 10^{-47}) with *PHEX* and *SMS* as annotated genes.

Fine-mapping of 3 top loci

Due to the GWAS design, the most strongly associated variants are likely to be in linkage disequilibrium (LD) with the causative variants rather than biologically causal themselves [30, 44]. Therefore, in order to refine the signals at the loci achieving the highest GWAS significance level, we proceeded to implement a statistical fine-mapping approach for the first three top hit signals through a Bayesian technique [21] that combines the genetic association and LD patterns to

identify variants with high posterior probabilities of being causal. This approach allows for the probability of multiple causal variants per region, avoiding the simplistic assumption of a single causal variant per locus, which is likely to be incorrect at many risk loci [45-47]. It is important to add that the results are conditional on the available data [46]. (Supplementary Tables 3A, 3B and 3C)

After selecting only directly genotyped SNPs or SNPs imputed with high certainty (see **Methods**), we were able to restrict the number of potential causative variants to a few in each of the three top loci. Variants with a posterior probability equal or larger than 0.8 [High Posterior Probability Variants (HPPVs); **Table 5**] were considered highly likely of being causal, as previously defined [46].

In the first top hit locus (6p21.31), two variants had a high probability of causality: a) 6:33658780 (rs35506178), an intragenic variant within *ITPR3*, a gene that encodes Inositol 1,4,5-triphosphate receptor type 3, a second messenger that mediates the release of intracellular calcium (probability of causality of 0.9999) and b) 6:33685417, an intergenic variant whose closest annotated gene is *IP6K3* (probability of causality of 0.9926). Of note, the sentinel SNP of this locus (6:33715837, rs9469580) had a rather low probability of causality (0.0033).

In the second top hit locus (12p13.32), four intergenic variants displayed a probability of 1 of being causal: a) 12:4493938 (rs720333), a variant with *FGF23* as annotated gene; b) 12:4562881 (rs1077434), a variant with *FGF6* as annotated gene, and c) 12:4596782 (rs71579224) and d) 12:4632967 (rs11063213), two variants with *C12orf4* as annotated gene.

In the third top hit locus (1p36.12), four variants displayed a high probability of causality: a) the sentinel SNP of this locus (1:21820042, rs12132412) is an intergenic variant with *ALPL* as annotated gene (probability of causality of 1); b) 1:21717027 (rs72657152), an intergenic variant with both *ALPL* and *NBPF3* as annotated genes (probability of causality of 0.999), and c) 1:21959015 (rs1557087) and d) 1:21944352 (rs748488), two intragenic variants within *RAP1GAP*, a gene

that encodes a GTPase activating protein that plays a key role in receptor signaling pathways (probability of causality of 0.999).

Discussion

This GWAS of serum phosphate levels in UKBB has shown a >20-fold increase in the number of loci related to serum phosphate levels in humans, from 10 to 182 loci. We were able to identify 264 independent autosomal SNPs clustered in 182 loci of which 172 are new. This includes the analysis of the X-chromosome that yielded three independent signals in men (clustered in two loci) and one signal in women.

Our SNP-based heritability estimates of ~12% suggest that serum phosphate level does not have a particularly high heritability due to common variants, as compared to the previous overall heritability estimated from twin studies of 53%. Yet, we now can explain ~7-8% of the overall variance by the identified SNPs. It is important to mention that our estimates of SNP-based heritability are probably underestimated. Residual stratification is possible despite the implementation of mixed models – though this has been described mostly for highly stratified traits [48, 49]. If that is the case for serum phosphate levels, more sophisticated methods for detecting and controlling population structure (chromosome painting or mixed models that includes environmental covariance) will be necessary to improve the inference [49, 50]. Current available models for partitioned heritability systematically exclude the MHC region, an important limitation considering our results.

Taken together, the combined SNPs we identified that determine serum phosphate level can form a polygenic risk score (PRS). Such a PRS is an important step towards identifying and stratifying subjects that might be prone to low or high serum phosphate levels, which is of clinical relevance. Yet, larger GWAS discovery studies are needed to be able to explain more of the variance to improve accuracy of such diagnostic tools. In addition, such a PRS might be used in Mendelian

Randomization studies to investigate, for example, causality of serum phosphate levels in relation to previously reported epidemiological associations with disease phenotypes and mortality.

For both the whole white European cohort and white British subset from UK Biobank, the top hit was shown to map to the Major Histocompatibility Complex, a finding that has not been previously explored.

The Major Histocompatibility Complex (MHC), which maps to 6p21.3, has traditionally been related to autoimmune and infectious diseases [51]. However, the genetic determinants of a wide variety of traits have gradually been mapped to this region, unraveling that its role outside autoimmunity has previously been severely underestimated. Indeed, the MHC is currently considered the region of the genome with the largest number of genetic associations not only for disease but also for quantitative traits [25]. The MHC includes the HLA region, the extended region and the flanking region [43]. The HLA region includes classical and non-classical HLA genes. The extended and flanking regions include other genes, progressively shown to be associated to a growing number of genetic associations. Importantly, the MHC region is the most polymorphic region of the genome, displays the strongest level of LD of the genome, has long range haplotypes and, in addition, has been under strong selection pressure [52, 53]. As a consequence, highly population-specific patterns of MHC architecture have arisen through evolution and genetic associations within MHC are highly susceptible to stratification.

By far, the strongest level of evidence of association of serum phosphate within the entire cohort of European-ancestry UK Biobank maps to 6p21.3, in the region flanking the MHC. Importantly, our results changed little when we restricted the analysis to a more genetically homogenous subset within the UKBB consisting of participants of White British ancestry, defined both as a self-reporting ancestry as such in addition to the results of a Bayesian algorithm designed to detect outliers and based on the first six PCs [16].

Hirata and colleagues [43] have recently extended the entire MHC to span the region from 24-36 Mb (GRCh 37/hg19). They have conducted a fine-mapping effort for the construction of a population-specific HLA imputation reference panel in a Japanese ancestry sample (n:1120) and reported a phosphate-related top hit in this region whose annotated gene is also *IP6K3*. The importance of population-specific HLA reference panels has been previously highlighted [53]. In addition, this reference panel was used to densely impute a large sample (n:166.190) from patients from BioBank Japan [54], through the implementation of the gold-standard SNP2HLA [55]. Importantly, the accuracy of this imputation effort reached high levels, which are mandatory for the success of fine-mapping efforts.

The Bayesian statistical fine-mapping approach that we implemented within the first three top hit loci strongly suggested a key role in serum phosphate levels for the following genes: *ITPR3, IP6K3, FGF23, FGF6, C12orf4, ALPL* and *RAP1GAP*. With the exception of *ALPL* and *FGF23*, these genes are novel in relation to determining phosphate levels and the underlying biological mechanisms have not been elucidated. Yet, the functional follow-up of the associated loci from this GWAS is beyond the scope of the current study. This will include further genetic fine mapping, determining overlap with GWAS hits for other traits and diseases, and molecular biological functional studies, to better understand -per such locus- which genetic variant(s) is (are) influencing the serum phosphate level, and through what mechanism. This will provide a rich source of novel biological insights in phosphate homeostasis which will be important for understanding distortions, and for the design of potential treatments.

Obviously, our results need replication in other datasets of European extraction, but also other ethnicities will be interesting to pursue in this respect. Replication of genetic associations whose results have been obtained through mixed models has been shown to be susceptible to an important decrease in power when the replication is intended through the standard meta-analytic approach. Bulik-Sullivan [56] has shown the importance of replicating findings from mixed models

in, ideally, equally-sized cohorts, and to pool the data instead of meta-analyzing the summary statistics. This condition is particular for mixed models and does not apply to linear regression models.

Trans-ethnic meta-analysis has the potential of providing better understanding of GWAS results [48]. Specifically for the MHC region, the trans-ethnic approach offers an increase in statistical power to identify associated loci in autoimmune diseases [57]. In particular, risk alleles in the HLA region have shown to share effect size and direction across Asian and European populations [20]. In addition, Morris [22] has shown that the inclusion of diverse ancestry groups in a meta-analysis is able to increase the power of fine-mapping to detect new variants through leveraging of local differences in LD patterns. This approach is more satisfactory than the implementation of both fixed and random effects.

In summary, we hereby identified 264 conditionally independent SNPs associated with serum phosphate levels in participants of European ancestry in UKBB. The strongest association signal mapped to the MHC region. Due to the implementation of mixed model approach, our results are not expected to be influenced by population stratification or relatedness. We found suggestive evidence for sex differences for three SNPs. Our preliminary fine-mapping results have confirmed the role of genes *FGF23* and *ALPL* in determining phosphate, but have also suggested a key role for several genes not known to influence phosphate homeostasis. Subsequent specialized fine-mapping techniques, especially in the MHC region, will identify credible sets of potentially causative SNPs, highly relevant to improve the physiologic knowledge on phosphate homeostasis and to perform functional studies. Through this work, we hope to be able to contribute to the future identification of genes suited for development of medications that may provide benefit for patients with disturbances in phosphate homeostasis including the growing number of patients with chronic kidney disease.

Table 1. Summary of results from GWAS of serum phosphate levels in the UK Biobank

Summary	**European ancestry cohort**	**White British ancestry cohort**
n	392.655	354.798
Number of independent SNPs	264	243
Number of genotyped SNPs	41	42
*Number of loci **	182	172
*Number of new loci ***	172	162
Number of loci in the X-chromosome	2 men / 1 women	2 men / 1 women
Total variance explained of P levels	7.62%	7.21%

*Locus defined as +/- 500 kb from top hit, according to [58].
Variance formula as described by Visscher et al [59].
** In comparison to GWAS from CHARGE & BBJ

Table 2a. Genomic control according to SNP subsets: European ancestry cohort

Software	λ	n_O SNPs
Easy Strata	1.199	19078819
GCTA	1.277	14073408
LD score	1.501	1198835
BOLT-LMM	1.345	586184*

*High-quality SNPs as implemented in the GRM

Table 2b. Genomic control and LD score results in the European ancestry cohort

	Standard LD score	*LD score baseline*
λ	1.50	1.49
*h2**	0.11	0.13
Intercept	1.10	1.07
Attenuation ratio	0.10	0.07

LD score baseline for partitioned heritability, as suggested for Biobank cohorts by the creators of BOLT-LMM. Attenuation ratio is considered appropriate if < 0.08.
*Undestimated value due to method

Table 3. Replication of previously reported GWAS hits for serum P levels in the UK Biobank

Chromosomal band	Top SNP: CHARGE	Top SNP: BioBank Japan	Top SNP: UK Biobank	Annotated gene (s)
1p36.12	rs1697421	rs1106357	rs12132412	*ALPL, NBPF3*
3q21.1	rs17265703	-	rs73186030	*CASR, CSTA*
5q35.3	rs4074995*	rs35716097	rs10051765	*RGS14, SLC34A1*
6p21.31	rs9469578	rs73743323	rs9469580	*IP6K3*
6q23.2	rs453639*	rs17060705	rs453639	*ENPP3*
6q23.3	rs947583	-	rs136093163	*PDE7B*
12p13.32	rs2970818	rs12368351	rs2970818	*FGF6,FGF23*
12p13.2	-	rs3903005	rs10743976	*ETV6*
17q24.2	-	rs35186465	rs11871728	*LINC01482, ABCA8*
Xp22.11	-	rs178710	rs178688	*PHEX*

*These SNPs did not reach nominal significance level at replication in CHARGE [14]

Table 4. Summary of conditionally independent signals for P levels located within the region flanking the MHC and annotated genes

Summary	European ancestry cohort (n=392.655)	White British ancestry cohort (n=354.798)
1.	6:33715837 (rs9469580); *IP6K3*	6:33715159 (rs60447213); *IP6K3*
2.	6:33445847 (rs143061808); *ZBTB9*	6:33445847 (rs143061808); *ZBTB9*
3.	6:33536670 (rs210132); *BAK1*	6:33029437 (rs6457710); *HLA-DPA1*
4.	6:33765465 (rs12153877); *MLN*	6:33017895 (rs112393700); *HLA-DOA*
5.	6:33821346 (rs9394173); *DQ591368*	6:33515520 (rs210152); *BAK1*
6.	6:33849073(rs12209273); *DQ591368*	6:33765465 (12153877); *MLN*
7.	6:34185755 (rs2797964); *HMGA1*	6:33849073(rs12209273); *DQ591368*
8.	6:25715657 (rs116009877); *SCGN*	6:34174908 (rs1776877); *HMGA1*
9.	6:31668049 (rs9267542); *ABHD16A*	6:34616322 (rs3800461); *C6orf106*
10.	-	6:25715657 (rs116009877); *SCGN*
11.	-	6:27151933 (rs59682554); *HIST1H2AH*
12.	-	6:31630665 (rs59345959); *GPANK1*
13.		6:31815723 (rs12210887); *SLC44A4*

The extended Major Histocompatibility Complex spans 24-36 Mb in 6p (GRCh37)

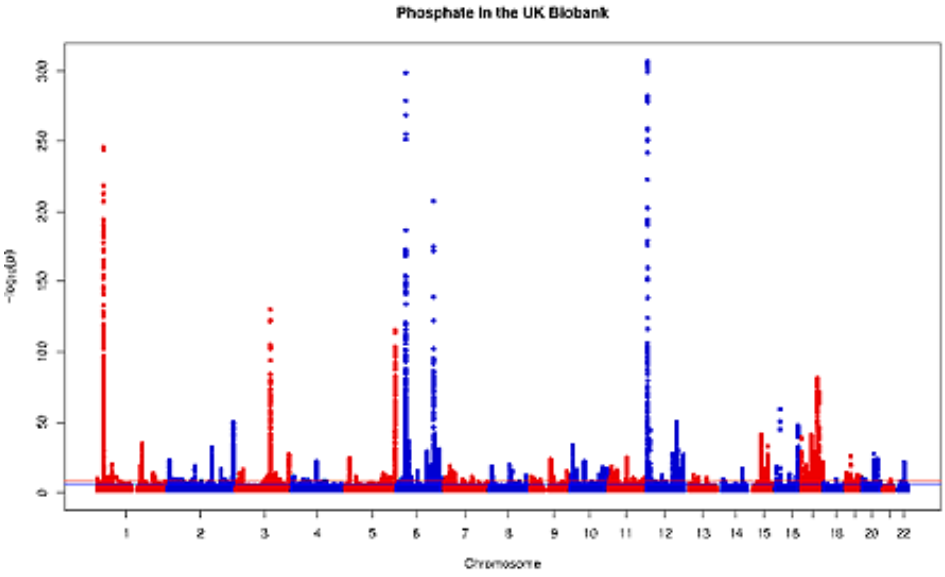
Table 5. Summary of High Posterior Probability Variants (HPPVs) from the first three top hit loci: results from a Bayesian fine-mapping approach

Locus	Chr:Pos	SNP rsID	PP
First top hit locus			
6p21.31	6:33658780	rs35506178	0.9999
6p21.31	6:33685417		0.9926
Second top hit locus			
12p13.32	12:4493938	rs720333	1
12p13.32	12:4562881	rs10774234	1
12p13.32	12:4596782	rs71579224	1
12p13.32	12:4632967	rs11063213	1
Third top hit locus			
1p36.12	1:21820042	rs12132412	1
1p36.12	1:21717027	rs72657152	0.9991
1p36.12	1:21944352	rs748488	0.9991
1p36.12	1:21959015	rs1557087	0.9991

PP: Posterior probability of causality

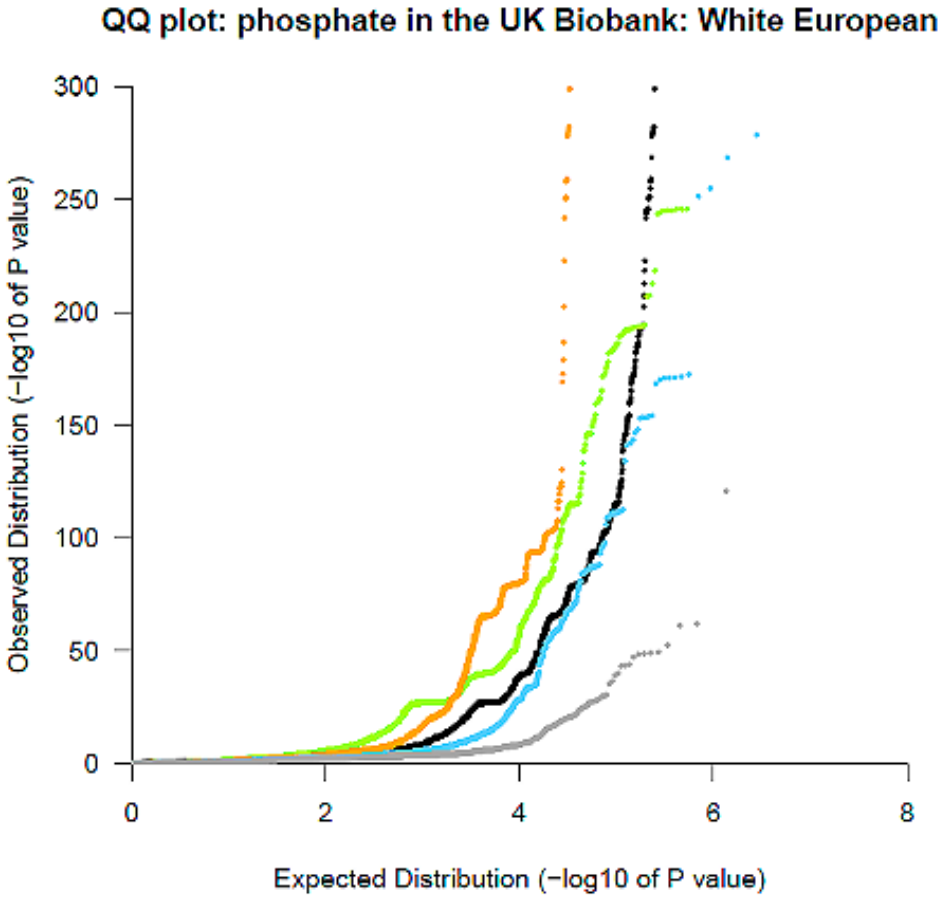
High posterior probability of causality is defined as PP $\geq$ 0.8 [46]

Figure 1. Manhattan plot for the UKBB GWAS of serum phosphate levels in the sex-combined cohort of white European ancestry subjects



Significance line at $p< 5x10^{-9}$; (red) suggestive line at $p< 5x10^{-6}$ (blue)

Figure 2. QQ plot for the UKBB GWAS of serum P levels in the white European



Genomic control inflation factors (λ) stratified according to minor allele frequency (MAF), as follows: all SNPs: **λ 1.15** (dark blue); SNPs with MAF >0.2 & <0.8: **λ 1.62** (green); SNPs with MAF >0.05 & <0.2: **λ 1.43** (orange); SNPs with MAF >0.01 & <0.05: **λ 1.20** (light blue); SNPs with MAF ≥0.005 & < 0.01: **λ 1.09** (grey).

Figure 3. Miami plot for serum P levels according to sex category in UKBB GWAS of the white European ancestry cohort

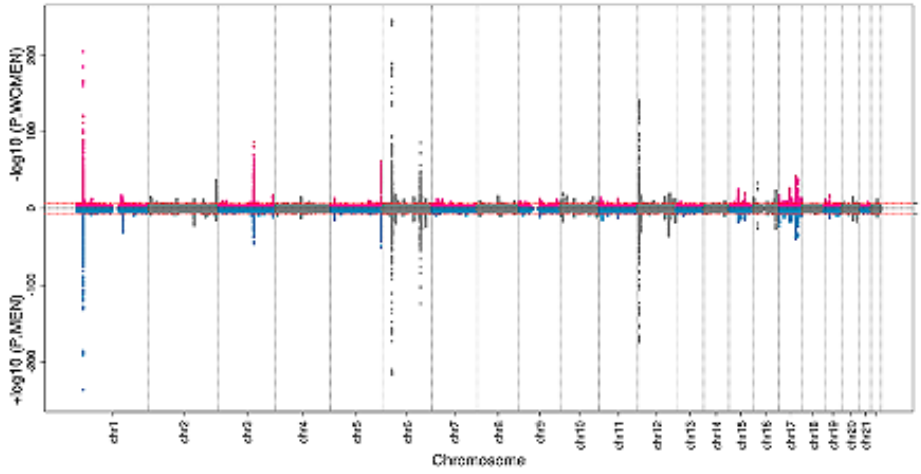


Figure 4. Regional plots for two top hits on chromosome 1 and 6 of the UKBB serum phosphate GWAS in subjects of white European ancestry

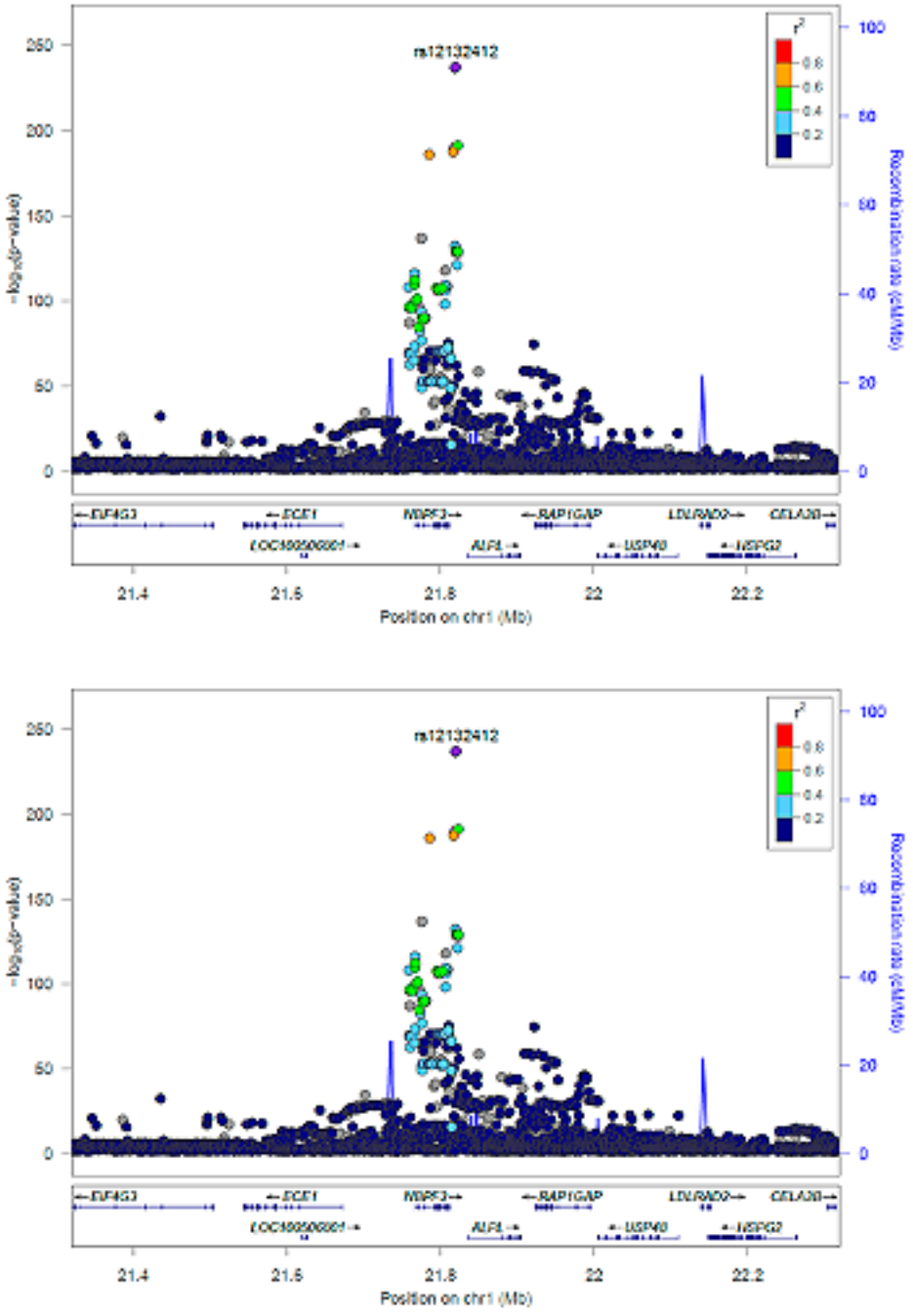
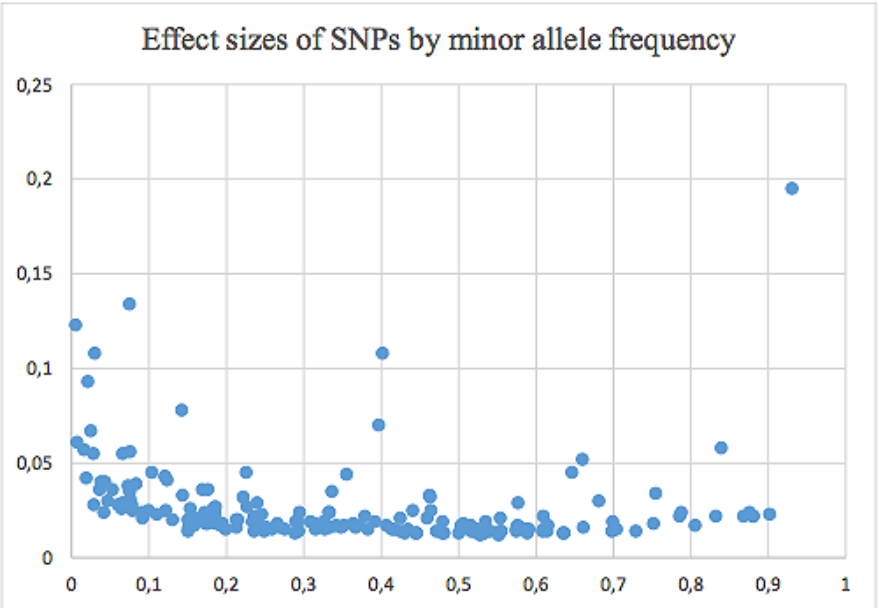


Figure 5. Scatterplot of the effect sizes (betas) by minor allele frequency for serum phosphate-related SNPs identified in the UKBB GWAS



References

1. **Berndt T, Kumar R**. Phosphatonins and the regulation of phosphate homeostasis. *Annu Rev Physiol* 2007; 69: 341-59.

2. **Zhang R et al**. Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J Bone Miner Res* 2011; 26(5):1047-56.

3. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK**. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998; 31(4): 607-17.

4. **Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Update Work Group**. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2017; 7(1): 1-59.

5. **Campos-Obando N et al.** Serum Phosphate Is Associated With Fracture Risk: The Rotterdam Study and MrOS. *J Bone Miner Res* 2017; 32(6): 1182-93.

6. **Shin S et al**. Impact of serum calcium and phosphate on coronary atherosclerosis detected by cardiac computed tomography. *Eur Heart J* 2012; 33(22): 2873-81.

7. **Onufrak SJ et al**. Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009; 169(1): 67-77.

8. **Dhingra R et al**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007; 167(9): 879-85.

9. **Campos-Obando N et al**. Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men. *Eur J Epidemiol* 2018; 33(9): 859-71.

10. **Bai W, Li J, Liu J**. Serum phosphorus, cardiovascular and all-cause mortality in the general population: A meta-analysis. *Clin Chim Acta* 2016; 461: 76-82.

11. **Hunter DJ et al**. Genetic contribution to renal function and electrolyte balance: a twin study. *Clin Sci (Lond)* 2002; 103(3): 259-65.

12. **ADHR Consortium**. Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nat Genet* 2000; 26(3): 345-8.

13. **Bastepe M, Jüppner H**. Inherited hypophosphatemic disorders in children and the evolving mechanisms of phosphate regulation. *Rev Endocr Metab* Disord 2008; 9(2): 171-80.

14. **Kestenbaum B et al**. Common genetic variants associate with serum phosphorus concentration. *J Am Soc Nephrol* 2010; 21(7): 1223-32.

15. **Kanai M et al.** Genetic analysis of quantitative traits in the Japanese population links cell types to complex human diseases. *Nat Genet* 2018; 50(3): 390-400.

16. **Bycroft C et al**. The UK Biobank resource with deep phenotyping and genomic data. *Nature* 2018; 562(7726): 203-9.

17. **Loh PR et al**. Mixed-model association for biobank-scale datasets. *Nat Genet* 2018; 50(7): 906-8.

18. **Loh PR et al**. Efficient Bayesian mixed-model analysis increases association power in large cohorts. *Nat Genet* 2015; 47(3): 284-90.

19. **Kichaev G, Pasaniuc B**. Leveraging Functional-Annotation Data in Trans-ethnic Fine-Mapping Studies. *Am J Hum Genet* 2015; 97(2): 260-71.

20. **Okada Y et al**. Risk for ACPA-positive rheumatoid arthritis is driven by shared HLA amino acid polymorphisms in Asian and European populations. *Hum Mol Genet* 2014; 23(25): 6916-26.

21. **Kichaev G et al**. Integrating functional data to prioritize causal variants in statistical fine-mapping studies. *PLoS Genet* 2014; 10(10): e1004722.

22. **Morris AP.** Transethnic meta-analysis of genomewide association studies. *Genet Epidemiol* 2011; 35(8): 809-22.

23. **Band G, Marchini J**. BGEN: a binary file format for imputed genotype and haplotype data. *bioRxiv* 2018: 308296.

24. **Howie B, Fuchsberger C, Stephens M, Marchini J**. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet* 2012; 44(8): 955-9.

25. **Trowsdale J, Knight JC**. Major histocompatibility complex genomics and human disease. *Annu Rev Genomics Hum Genet* 2013; 14: 301-23.

26. **Isakova T et al**. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011; 79(12): 1370-8.

27. **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.

28. **Lin DY.** A simple and accurate method to determine genomewide significance for association tests in sequencing studies. *Genet Epidemiol* 2019; 43(4): 365-72.

29. **Bulik-Sullivan BK et al.** LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat Genet* 2015; 47(3): 291-5.

30. **Spain SL, Barrett JC**. Strategies for fine-mapping complex traits. *Hum Mol Genet* 2015; 24(R1): R111-9.

31. **Eisenhart C**. The assumptions underlying the analysis of variance. *Biometrics* 1947; 3(1): 1-21.

32. **Henderson CR**. Best linear unbiased estimation and prediction under a selection model. *Biometrics* 1975; 31(2): 423-47.

33. **Yang J, Zaitlen NA, Goddard ME, Visscher PM, Price AL**. Advantages and pitfalls in the application of mixed-model association methods. *Nat Genet* 2014; 46(2): 100-6.

34. **Listgarten J, Lippert C, Kadie CM, Davidson RI, Eskin E, Heckerman D**. Improved linear mixed models for genome-wide association studies. *Nat Methods* 2012; 9(6): 525-6.

35. **Zhang Y, Qi G, Park JH, Chatterjee N**. Estimation of complex effect-size distributions using summary-level statistics from genome-wide association studies across 32 complex traits. *Nat Genet* 2018. 50(9): 1318-26.

36. **Fisher RA**. The Correlation between Relatives on the Supposition of Mendelian Inheritance. *Transactions of the Royal Society of Edinburgh* 1918; 52: 399-433.

37. **Devlin B, Roeder K.** Genomic control for association studies. *Biometrics* 1999; 55: 997- 1004.

38. **Yang J et al.** Genomic inflation factors under polygenic inheritance. *Eur*

J Hum Genet 2011; 19(7): 807-12.

39. **Gazal S et al.** Linkage disequilibrium-dependent architecture of human complex traits shows action of negative selection. *Nat Genet* 2017; 49(10): 1421-7.

40. **Stahl EA et al**. Bayesian inference analyses of the polygenic architecture of rheumatoid arthritis. *Nat Genet* 2012; 44(5): 483-9.

41. **Astle WJ et al**. The Allelic Landscape of Human Blood Cell Trait Variation and Links to Common Complex Disease. *Cell* 2016; 167(5): 1415-29.

42. **Winkler TW, Justice AE, Cupples LA, Kutalik Z, Heid IM and the GIANT Consortium**. Approaches to detect genetic effects that differ between two strata in genome-wide meta-analyses: Recommendations based on a systematic evaluation. *PLoS One* 2017; 12(7): e0181038.

43. **Hirata J et al**. Genetic and phenotypic landscape of the major histocompatibilty complex region in the Japanese population. *Nat Genet* 2019; 51(3): 470-80.

44. **Schaid DJ, Chen W, and Larson NB.** From genome-wide associations to candidate causal variants by statistical fine-mapping. *Nat Rev Genet* 2018; 19(8): 491-504.

45. **Hormozdiari F, Kostem E, Kang EY, Pasaniuc B, Eskin E.** Identifying causal variants at loci with multiple signals of association. *Genetics* 2014; 198(2): 497-508.

46. **Fachal L et al.** Fine-mapping of 150 breast cancer risk regions identifies 191 likely target genes. *Nat Genet* 2020; 52(1): 56-73.

47. **Dadaev T et al**. Fine-mapping of prostate cancer susceptibility loci in a large meta-analysis identifies candidate causal variants. *Nat Commun* 2018; 9(1): 2256.

48. **Lawson DJ et al**. Is population structure in the genetic biobank era irrelevant, a challenge, or an opportunity? *Hum Genet* 2020; 139(1): 23-41.

49. **Haworth S et al.** Apparent latent structure within the UK Biobank sample has implications for epidemiological analysis. *Nat Commun* 2019; 10(1): 333.

50. **Heckerman D et al.** Linear mixed model for heritability estimation that explicitly addresses environmental variation. *Proc Natl Acad Sci USA* 2016;

113(27): 7377-82.

51. **de Bakker PI et al.** A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38(10): 1166-72.

52. **Kawashima M, Ohashi J, Nishida N, Tokunaga K**. Evolutionary analysis of classical HLA class I and II genes suggests that recent positive selection acted on DPB1*04:01 in Japanese population. *PLoS One* 2012; 7(10): e46806.

53. **Okada Y et al.** Construction of a population-specific HLA imputation reference panel and its application to Graves' disease risk in Japanese. *Nat Genet* 2015; 47(7): 798-802.

54. **Nagai A et al**. Overview of the BioBank Japan Project: Study design and profile. *J Epidemiol* 2017; 27(3 Suppl): S2-S8.

55. **Jia X et al**. Imputing amino acid polymorphisms in human leukocyte antigens. *PLoS One* 2013; 8(6): p. e64683.

56. **Bulik-Sullivan B**. Mixed models for meta-analysis and sequencing. 2015 doi: https://doi.org/10.1101/020115.

57. **Okada Y et al.** Genetics of rheumatoid arthritis: 2018 status. *Ann Rheum Dis* 2019; 78(4): 446-53.

58. **Wood AR et al**. Defining the role of common variation in the genomic and biological architecture of adult human height. *Nat Genet* 2014. 46(11): 1173-86.

59. **Visscher PM et al.** 10 Years of GWAS Discovery: Biology, Function, and Translation. *Am J Hum Genet* 2017; 101(1): 5-22.

Supplemental Tables

1a. Summary of genome-wide significant loci associated with P levels in UKBB participants with European ancestry.

1b. Summary of genome-wide significant loci associated with P levels in UKBB participants with White British ancestry.

2. SNPs with suggestive p value for sex difference levels in UKBB participants with European ancestry.

3a. Summary of results from fine-mapping approach in the first top hit locus (6p21.31).

3b. Summary of results from fine-mapping approach in the second top hit locus (12p13.32).

3c. Summary of results from fine-mapping approach in the third top hit locus (1p36.12).

Suppl. Table 1a. Summary of genome-wide significant loci associated with P levels in UKBB participants with European ancestry

Chrom. band	Locus ID	Sentinel SNP	rsID	*Closest gene (s)*	Lowest p	# SNPs
Chromosome 1: 30 independent primary signals clustered in 14 loci						
1p36.12	**chr1:1**	**1:21820042**	**rs12132412**	***ALPL, NBPF3***	**1.2 e -194**	**14**
1q23.2	chr1:2	1:159578143	rs77672383	*APCS*	3.9 e -33	2
1p32.3	chr1:3	1:51760147	rs41287288	*TTC39A*	2.5 e -21	1
1q22	chr1:4	1:155197462	rs2990245	*GBAP1*	1.0 e -18	1
1q32.1	chr1:5	1:199007208	rs7529925	*LINC01221*	3.7 e -14	1
1p34.2	chr1:6	1:43435904	rs841571	*WDR65,EBNA1BP2*	8.0 e -13	2
1p34.2	chr1:7	1:42040740	rs941972	*HIVEP3*	8.1 e -13	1
1p36.22	chr1:8	1:12042506	rs4845891	*MFN2*	1.4 e -11	1
1q32.1	chr1:9	1:205476001	rs1538369	*CDK18*	3.4 e -11	2
1p31.3	chr1:10	1:68277744	rs12409158	*GNG12*	4.1 e -11	1
1p36.33	chr1:11	1:1065296	rs4072537	*C1orf159*	2.5 e -10	1
1p31.1	chr1:12	1:78450517	rs34517439	*DNAJB4*	5.6 e -10	1
1p36.11	chr1:13	1:26489633	rs7546500	*FAM1101*	8.6 e -10	1
1p36.11	chr1:14	1:27668610	rs41291086	*SYTL1*	1.0 e -9	1
Chromosome 2: 18 independent primary signals clustered in 13 loci						
2q37.1	chr2:1	2:234296650	rs838718	*DGKD*	3.3 e -46	4
2q24.1	chr2:2	2:158113579	rs77149268	*GALNT5*	1.3 e -31	1
2p25.1	chr2:3	2:7150527	rs445411	*RNF144A*	4.9 e -23	2
2q11.2	chr2:4	2:97437990	rs878919	*CNNM4*	8.2 e -20	1
2q32.2	chr2:5	2:191281320	rs36064835	*MFSD6*	2.5 e -16	1
2p25.1	chr2:6	2:10094329	rs3828261	*GRHL1*	2.0 e -12	2
2q33.1	chr2:7	2:198583267	rs4850438	*BOLL*	1.4 e -10	1
2p16.3	chr2:8	2:48548442	rs13385535	*FOXN2*	1.9 e -10	1
2p23.3	chr2:9	2:27651375	rs2303370	*NRBP1*	3.8 e -10	1
2p23.3	chr2:10	2:25534999	rs7596024	*DNMT3A*	6.6 e -10	1
2q36.3	chr2:11	2:230111334	rs55923451	*PID1*	8.1 e -10	1
2q33.3	chr2:12	2:207306586	rs76436488	*ADAM23*	1.0 e -9	1
2p11.2	chr2:13	2:85410933	rs2568203	*TCF7L1*	2.8 e -9	1
Chromosome 3: 13 independent primary signals clustered in 9 loci						
3q21.1	chr3:1	3:122013465	rs73186030	*CASR, CSTA,CCDC58*	9.3 e -145	5
3q28	chr3:2	3:188400557	rs56328339	*LPP*	2.9 e -28	1
3p24.2	chr3:3	3:25404136	rs13325064	*RARB*	7.6 e -17	1
3p25.2	chr3:4	3:12389313	rs17036326	*PPARG*	1.5 e -14	1
3q13.31	chr3:5	3:114289172	rs114712971	*ZBTB20*	2.4 e -13	1
3q23	chr3:6	3:141133450	rs1991431	*ZBTB38*	2.7 e -13	1

3q13.2	chr3:7	3:113304781	rs9827928	*SIDI1*	6.0 e -12	1
3q22.3	chr3:8	3:135773661	rs28576629	*PPP2R3A*	1.0 e -10	1
3q13.12	chr3:9	3:107235109	rs34040779	*BBX, LINC01990*	3.6 e -9	1
Chromosome 4: 7 independent primary signals clustered in 6 loci						
4q22.1	chr4:1	4:88587881	rs1462373	*DMP1*	8.5 e -22	2
4p16.1	chr4:2	4:7759976	rs11737447	*AFAP1-AS1*	9.0 e -12	1
4p16.3	chr4:3	4:3478051	rs2699425	*DOK7*	1.7 e -10	1
4p14	chr4:4	4:40563796	rs6828311	*RBM47*	4.5 e -10	1
4q28.1	chr4:5	4:124437792	rs1993190	*SPRY1*	2.1 e -9	1
4q26	chr4:6	4:115458168	rs11938628	*UGT8*	4.3 e -9	1
Chromosome 5: 16 independent primary signals clustered in 8 loci						
5q35.3	chr5:1	5:176799992	rs10051765	*RGS14*	1.7 e -27	3
5p15.2	chr5:2	5:14984166	rs706299	*ANKH,LINC02149*	1.7 e -23	6
5q31.1	chr5:3	5:133849687	rs6860961	*BCO32795,LINC01843*	5.8 e -14	1
5p13.2	chr5:4	5:36634332	rs12518871	*SLC1A3*	4.5 e -12	1
5q31.3	chr5:5	5:141713195	rs62383568	*LOC10192641*	1.0 e -11	1
5q31.1	chr5:6	5:131564501	rs111547866	*P4HA2*	1.4 e -11	1
5q33.3	chr5:7	5:158504140	rs7726961	*EBF1*	3.2 e -11	2
5q31.3	chr5:8	5:139637565	rs13161154	*PFDN1,CYSTM1*	7.2 e -11	1
Chromosome 6: 34 independent primary signals clustered in 17 loci						
6p21.31	**chr6:1**	**6:33715837**	**rs9469580**	***IP6K3***	**1.6 e -468**	**7**
6q23.2	chr6:2	6:132049657	rs453639	*ENPP3*	5.8 e -135	6
6q23.3	chr6:3	6:136093163	rs4385331	*LINC00271,PDE7B*	2.0 e -35	2
6p21.1	chr6:4	6:44***121	rs112748697	*SUPT3H*	1.7 e -30	3
6q21	chr6:5	6:108944165	rs3813498	*FOXO3*	2.4 e -30	1
6q25.1	chr6:6	6:152010561	rs2982572	*ESR1*	1.8 e -19	3
6q22.33	chr6:7	6:127118646	rs35848181	*AK127472*	5.5 e -19	1
6q23.2	chr6:8	6:134520896	rs1743955	*SGK1*	3.2 e -18	1
6q13	chr6:9	6:74486371	rs4263551	*CD109*	9.3 e -17	1
6p21.33	chr6:10	6:31668049	rs9267542	*ABHD16A*	4.4 e -15	1
6q23.3	chr6:11	6:137259725	rs12191772	*SLC35D3,PEX7,NHEG1*	3.5 e -12	1
6p22.2	chr6:12	6:25715657	rs116009877	*SCGN,SLC17A4*	5.5 e -12	1
6q13	chr6:13	6:72197034	rs573205	*LINC00472,LINC01626*	6.0 e -11	1
6q26	chr6:14	6:163854303	rs814143	*QK1*	1.8 e -10	2
6q22.31	chr6:15	6:121787498	rs9375033	*GJA1*	2.1 e -10	1
6p12.2	chr6:16	6:52735206	rs9474335	*GSTA5,GSTA3*	3.2 e -10	1
6p22.3	chr6:17	6:18914211	rs912981	*MIR548A1,AK097585*	7.1 e -10	1

Chromosome 7: 9 independent primary signals clustered in 8 loci						
7p21.1	chr7:1	7:20051287	rs13328356	*LOC101927668*	2.7 e -19	1
7p15.1	chr7:2	7:28479848	rs6462085	*CREBS*	5.3 e -17	2
7p21.1	chr7:3	7:17284577	rs4410790	*AHR*	2.1 e -14	1
7p14.1	chr7:4	7:38152697	rs2177470	*STARD3NL,EPDR1*	2.7 e -13	1
7q21.3	chr7:5	7:97929081	rs13232861	*BAIAP2L1*	2.0 e -12	1
7p22.1	chr7:6	7:6104265	rs112367067	*USP42,EIF2AK1*	2.8 e -11	1
7q22.1	chr7:7	7:101939127	rs2242581	*SH2B2*	3.4 e -10	1
7q11.21	chr7:8	7:65491123	rs2460421	*ASL,GUSB,CRCP*	5.2 e -10	1
Chromosome 8: 6 independent primary signals clustered in 5 loci						
8q13.3	chr8:1	8:72507296	rs6983239	*EYA1,LOC100132641*	3.0 e -20	1
8p23.1	chr8:2	8:8198306	rs4840337	*SGK223,PRAG1*	9.5 e -17	1
8q21.13	chr8:3	8:82670771	rs35094336	*CHMP4C*	5.8 e -16	1
8q21.11	chr8:4	8:76478616	rs2941483	*HNF4G*	9.3 e -15	1
8q24.21	chr8:5	8:129950004	rs13264707	*LINC00824,MIR1208*	2.1 e -11	2
Chromosome 9: 11 independent primary signals clustered in 10 loci						
9q21.11	chr9:1	9:71461815	rs10869403	*PIP5K1B*	2.0 e -23	2
9q33.3	chr9:2	9:129294976	rs10819178	*MVB12B*	8.2 e -15	1
9q21.13	chr9:3	9:77499796	rs11144134	*TRPM6*	6.7 e -13	1
9q34.3	chr9:4	9:140130606	rs28542318	*SLC34A3*	1.3 e -12	1
9p24.3	chr9:5	9:908583	rs1033832	*DMRT1*	4.6 e -12	1
9q34.11	chr9:6	9:130681892	rs4837205	*ST6GALNAC4,PIP5KL1*	1.8 e -11	1
9p24.1	chr9:7	9:4746539	rs447124	*AK3,RCL1*	1.1 e -10	1
9q21.2	chr9:8	9:80702276	rs4877397	*CEP78,GNAQ*	2.1 e -10	1
9p23	chr9:9	9:14017425	rs73648449	*NF1B*	7.4 e -10	1
9p13.3	chr9:10	9:33291520	rs830575	*NFX1*	2.0 e -9	1
Chromosome 10: 14 independent primary signals clustered in 11 loci						
10p14	chr10:1	10:9321916	rs111817798	*LOC10192672,LINC00709*	2.2 e -36	2
10q11.23	chr10:2	10:50313621	rs4509682	*VSTM4*	1.5 e -23	1
10q25.3	chr10:3	10:116080355	rs2420055	*AFAP1L2*	4.8 e -18	1
10q22.3	chr10:4	10:130903232	rs10764844	*PDXK*	6.6 e -17	1
10q24.2	chr10:5	10:101829514	rs61751507	*CPN1*	3.1 e -16	2
10p12.31	chr10:6	10:22280664	rs6650130	*DNAJC1*	8.7 e -16	1
10q24.32	chr10:7	10:104707727	rs56192823	*CNNM2*	1.1 e -15	2
10q26.13	chr10:8	10:126419743	rs12247543	*FAM53B*	1.9 e -14	1
10p14	chr10:9	10:8090380	rs1976684	*GATA3-AS1*	3.2 e -14	1
10p15.1	chr10:10	10:5254188	rs7897431	*AKR1C4*	1.8 e -13	1
10q24.2	chr10:11	10:100179851	rs2296436	*HPS1*	1.3 e -9	1

Chromosome 11: 9 independent primary signals, 9 loci						
11q13.1	chr11:1	11:65337251	rs1346	*SSSCA1-AS1*	3.3 e -24	1
11p15.3	chr11:2	11:11436233	rs56002986	*GALNT18*	1.4 e -19	1
11p14.1	chr11:3	11:30883876	rs273587	*DCDC5*	2.2 e -17	1
11p15.2	chr11:4	11:13550209	rs10832053	*PTH*	3.1 e -16	1
11q24.2	chr11:5	11:126225876	rs112771035	*ST3GAL4*	1.5 e -11	1
11q14.2	chr11:6	11:87901236	rs302650	*RAB38*	1.6 e -11	1
11p15.4	chr11:7	11:5573701	rs2647602	*HBG2*	2.6 e -11	1
11q21	chr11:8	11:95308854	rs11021221	*FAM76B,CEP57,SESN3*	6.8 e -10	1
11p13	chr11:9	11:33804010	rs11032385	*FBX03*	4.5 e -9	1
Chromosome 12: 27 independent primary signals clustered in 14 loci						
12p13.32	**chr12:1**	**12:4606168**	**rs2970818**	***C12orf4,FGF23,FGF6***	**1.2 e -272**	**11**
12p13.2	chr12:2	12:12166475	rs10743976	*ETV6*	1.7 e -53	2
12q23.3	chr12:3	12:107341385	rs7974499	*C12orf23,TMEM263*	1.8 e -51	1
12q22	chr12:4	12:94311934	rs74737968	*LOC105369911*	4.3 e -32	2
12q24.12	chr12:5	12:111884608	rs3184504	*SH2B3*	1.1 e -29	1
12q24.33	chr12:6	12:131582952	rs12579593	*GPR133*	1.1 e -27	1
12q23.3	chr12:7	12:108996922	rs34826644	*TMEM119,SELPLG*	1.4 e -22	1
12q24.31	chr12:8	12:121416864	rs1800574	*HNF1A*	7.6 e -18	1
12q24.31	chr12:9	12:123560731	rs596940	*PITPNM2*	2.9 e -16	2
12q13.3	chr12:10	12:56860020	rs2939302	*MIP*	1.0 e -13	1
12q24.31	chr12:11	12:122520575	rs61952922	*MLXIP*	8.0 e -12	1
12q24.13	chr12:12	12:113563105	rs35026779	*RASAL1*	2.3 e -11	1
12p12.2	chr12:13	12:21254963	rs4762813	*SLCO1B1,SLCO1B7*	8.8 e -11	1
12q14.1	chr12:14	12:58117737	rs238517	*OS9,AGAP2*	7.0 e -10	1
Chromosome 13: 4 independent primary signals, 4 loci						
13q13.1	chr13:1	13:33509030	rs7324259	*LINC00423*	6.1 e -13	1
13q14.3	chr13:2	13:51385422	rs1570603	*DLEU7,DLEU7-AS1*	4.6 e -11	1
13q31.1	chr13:3	13:80749431	rs9574586	*SPRY2*	1.1 e -10	1
13q14.11	chr13:4	13:42951449	rs9533090	*AKAP11,LOC105370177*	1.2 e -9	1
Chromosome 14: 4 independent primary signals clustered in 2 loci						
14q32.11	chr14:1	14:91463383	rs1286153	*RPS6KA5*	5.0 e -17	2
14q11.2	chr14:2	14:23774916	rs71413981	*BCL2L2*	3.0 e -10	2
Chromosome 15: 7 independent primary signals clustered in 5 loci						
15q21.2	chr15:1	15:51104350	rs12909505	*AK091906,SPPL2A*	2.3 e -38	2
15q24.1	chr15:2	15:75079474	rs34933034	*CSK*	2.1 e -22	2
15q22.2	chr15:3	15:60883281	rs339969	*RORA*	4.4 e -17	1
15q21.3	chr15:4	15:58674051	rs62001736	*UPC*	1.8 e -10	1
15q22.31	chr15:5	15:64159259	rs12594100	*HERC1,DAPK2*	4.4 e -10	1

Chromosome 16: 8 independent primary signals clustered in 7 loci						
16p13.11	chr16:1	16:16259596	rs41278174	*ABCC6*	4.8 e -58	1
16q23.2	chr16:2	16:79755446	rs4575545	*MAFTRR*	7.4 e -54	2
16p13.3	chr16:3	16:4016676	rs2230742	*ADCY9*	1.4 e -18	1
16q12.1	chr16:4	16:49888931	rs9745989	*ZNF423*	7.7 e -14	1
16q24.2	chr16:5	16:88526568	rs7206518	*ZFPM1*	2.7 e -13	1
16q22.3	chr16:6	16:73024276	rs1858800	*ZFHX3*	4.9 e -12	1
16q23.2	chr16:7	16:81534790	rs2925979	*CMIP*	2.1 e -10	1
Chromosome 17: 19 independent primary signals clustered in 16 loci						
17q24.2	chr17:1	17:66703728	rs11871728	*LINC01482,ABCA8*	2.0 e -69	2
17q12	chr17:2	17:37504933	rs8069451	*FBXL20*	9.6 e -44	1
17p13.3	chr17:3	17:1618363	rs11078597	*MIR22HG, ABR*	3.8 e -39	2
17q23.2	chr17:4	17:58996208	rs2120222	*BCAS3*	2.1 e -36	2
17q21.31	chr17:5	17:43946318	rs753236	*MAPT-AS1*	2.7 e -32	1
17q25.3	chr17:6	17:76930571	rs7213976	*TIMP2, LGALS3BP*	2. 9 e -22	1
17q21.32	chr17:7	17:45763005	rs4794047	*KPNB1,TBKBP1*	2.2 e -18	1
17p11.2	chr17:8	17:17947710	rs2955382	*GID4*	3.1 e -18	1
17q23.1	chr17:9	17:57836767	rs72838857	*VPM1*	2.3 e -15	1
17q11.2	chr17:10	17:29726603	rs4795607	*RAB11FIP4*	1.1 e -13	1
17q21.31	chr17:11	17:42090587	rs228778	*TMEM101*	2.6 e -13	1
17p13.1	chr17:12	17:10033679	rs11656696	*GAS7*	4.6 e -12	1
17p13.1	chr17:13	17:7213579	rs73977632	*EIF5A*	2.4 e -11	1
17q21.32	chr17:14	17:47005588	rs1057902	*UBE2Z*	3.2 e -10	1
17q22	chr17:15	17:53361838	rs1477141	*HLF*	1.7 e -9	1
17q21.33	chr17:16	17:47928342	rs79049182	*TAC4, FLJ45513*	2.1 e -9	1
Chromosome 18: 1 independent primary signal						
18q21.1	chr18:1	18:44749884	rs2684837	*SKOR2*	4.3 e -10	1
Chromosome 19: 12 independent primary signals, 12 loci						
19p13.11	chr19:1	19:17504801	rs34466478	*BSJ2,AK311380*	4.4 e -27	1
19p13.11	chr19:2	19:18575193	rs78030362	*ELL*	3.3 e -20	1
19p13.3	chr19:3	19:3102748	rs308032	*GNA11*	1.3 e -15	1
19q13.2	chr19:4	19:41839631	rs8105161	*TGFB1*	6.5 e -13	1
19q13.41	chr19:5	19:53405497	rs10401230	*ZNF888,ZNF320*	1.1 e -12	1
19q13.31	chr19:6	19:43826203	rs79338083	*CD177,PSG9*	3.4 e -12	1
19p13.12	chr19:7	19:14172921	rs10415758	*PALM3,MISP3,LOC113230*	1.1 e -11	1
19p13.2	chr19:8	19:11942362	rs427880	*ZNF440*	5.5 e -11	1
19p13.11	chr19:9	19:19717056	rs73004967	*PBXY*	2.0 e -10	1
19q13.2	chr19:10	19:38817628	rs35496032	*KCNK6*	4.9 e -10	1
19p13.3	chr19:11	19:4190185	rs77053710	*ANKRD24*	7.3 e -10	1
19q13.33	chr19:12	19:50044741	rs113886122	*RCN3*	4.0 e -9	1

Chromosome 20: 10 independent primary signals clustered in 7 loci						
20q12	chr20:1	20:39344272	rs3091842	*MAFB,TOP1*	1.3 e -32	3
20q13.2	chr20:2	20:52732362	rs17216707	*BCAS1,CYP24A1*	1.9 e -25	2
20q13.12	chr20:3	20:45569796	rs6124901	*EYA2*	4.2 e -11	1
20p12.3	chr20:4	20:8129491	rs8121126	*PLCB1*	3.1 e -10	1
20p13	chr20:5	20:1923972	rs6136492	*SIRPA,LOC727993*	3.6 e -10	1
20q13.13	chr20:6	20:48978609	rs11699816	*LOC264751,LINC01270*	7.2 e -10	1
20q13.12	chr20:7	20:43042364	rs1800961	*HNF4A*	7.8 e -10	1
Chromosome 21: 1 independent primary signal						
21q22.13	chr21:1	21:37835648	rs219770	*CLDN14*	3.6 e -9	1
Chromosome 22: 1 independent primary signal						
22q13.1	chr22:1	22:38599857	rs140916674	*MAFF*	4.7 e -22	1
Chromosome X, men: 3 independent primary signals clustered in 2 loci						
Xp22.12	chrXm:1	X:22039936	rs178676	*PHEX*	1.6 e -71	2
Xp22.31	chrXm:2	X:8902853	rs5934503	*FAM9A, FAM9B*	4.1 e -16	1
Chromosome X, women: 1 independent primary signal						
Xp22.11	chrXw:1	X:22043421	rs178688	*PHEX, SMS*	9.6 e -47	1

Chromosomal band and positioning were established according to GRCh 37 assembly. Sentinel SNP: independent SNP with lowest *p* value in locus. Lowest *p*: lowest *p* value in locus. # SNPs: number of independent SNPs associated with P levels per locus. The three top hit SNPs are highlighted in bold. ChrXm: locus in X-chromosome in men; chrXw: locus in X-chromosome in women

Suppl. Table 1b. Summary of genome-wide significant loci associated with P levels in UKBB participants with White British ancestry

Chrom. band	Locus ID	Sentinel SNP	rsID	Closest gene (s)	Lowest *p*	# SNPs
Chromosome 1: 26 independent primary signals clustered in 13 loci						
1p36.12	**chr1:1**	**1:21820042**	**rs12132412**	***ALPL, NBPF3***	**1.2 e -204**	**12**
1q23.2	chr1:2	1:159578143	rs77672383	*APCS*	8.4 e -31	2
1p32.3	chr1:3	1:51760147	rs41287288	*TTC39A*	8.1 e -21	1
1q22	chr1:4	1:155175390	rs370545	*THBS3*	3.6 e -15	1
1q32.1	chr1:5	1:199007208	rs7529925	*LINC01221*	1.0 e -12	1
1p34.2	chr1:6	1:42040740	rs941972	*HIVEP3*	1.4 e -11	1
1p34.2	chr1:7	1:43702316	rs3862225	*WDR65,EBNA1BP2*	3.4 e -11	2
1p31.3	chr1:8	1:68277744	rs12409158	*GNG12*	5.2 e -11	1
1q32.1	chr1:9	1:205482946	rs1538369	*CDK18*	6.8 e -11	1
1p36.22	chr1:10	1:12042443	rs4846082	*MFN2*	2.1 e -10	1
1p31.1	chr1:11	1:78743005	rs71641333	*MGC27382*	4.8 e -10	1
1p36.33	chr1:12	1:1065296	rs4072537	*C1orf159*	5.0 e -10	1
1p36.11	chr1:13	1:26522723	rs12732367	*CATSPER4*	7.6 e -10	1
Chromosome 2: 17 independent primary signals clustered in 12 loci						
2q37.1	chr2:1	2:234296444	rs838717	*DGKD*	3.9 e -41	4
2q24.1	chr2:2	2:158113579	rs77149268	*GALNT5*	1.8 e -28	1
2p25.1	chr2:3	2:7150527	rs445411	*RNF144A*	4.4 e -19	2
2q11.2	chr2:4	2:97437990	rs878919	*CNNM4*	2.7 e -18	1
2q32.2	chr2:5	2:191314604	rs111496708	*MFSD6*	8.8 e -15	1
2p23.3	chr2:6	2:27651375	rs2303370	*NRBP1*	1.4 e -11	1
2p25.1	chr2:7	2:10094329	rs3828261	*GRHL1*	7.1 e -11	2
2q33.1	chr2:8	2:198825668	rs10497809	*PLCL1*	3.7 e -10	1
2q36.3	chr2:9	2:230102422	rs954922	*PID1*	3.8 e -10	1
2p23.3	chr2:10	2:25534999	rs7596024	*DNMT3A*	1.1 e -9	1
2p16.3	chr2:11	2:48548442	rs13385535	*FOXN2*	1.9 e -9	1
2q33.3	chr2:12	2:207306586	rs76436488	*ADAM23*	4.9 e -9	1
Chromosome 3: 13 independent primary signals clustered in 9 loci						
3q21.1	chr3:1	3:122013465	rs73186030	*CASR, CSTA,CCDC58*	1.4 e -109	5
3q28	chr3:2	3:188400557	rs56328339	*LPP*	4.6 e -25	1
3q13.31	chr3:3	3:114289172	rs114712971	*ZBTB20*	3.7 e -13	1
3p24.2	chr3:4	3:25398610	rs7637258	*RARB*	7.2 e -13	1
3p25.2	chr3:5	3:12393125	rs1801282	*PPARG*	3.1 e -12	1
3q23	chr3:6	3:141142691	rs6440006	*ZBTB38*	3.9 e -12	1
3q13.2	chr3:7	3:113300183	rs2271494	*SIDT1*	1.1 e -10	1
3q22.3	chr3:8	3:135773661	rs28576629	*PPP2R3A*	5.0 e -10	1

3q13.12	chr3:9	3:107219309	rs34139997	*BBX, LINC01990*	3.0 e -9	1
Chromosome 4: 5 independent primary signals clustered in 4 loci						
4q22.1	chr4:1	4:88715259	rs7672820	*DMP1,IBSP,MEPE*	1.2 e -12	2
4p14	chr4:2	4:40563796	rs6828311	*RBM47*	4.0 e -10	1
4p16.3	chr4:3	4:3478051	rs2699425	*DOK7*	8.7 e -10	1
4q28.1	chr4:4	4:124437792	rs1993190	*SPRY1*	2.1 e -9	1
Chromosome 5: 13 independent primary signals clustered in 8 loci						
5q35.3	chr5:1	5:176799992	rs10051765	*RGS14*	2.3 e -108	2
5p15.2	chr5:2	5:14984166	rs706299	*ANKH,LINC02149*	1.2 e -24	4
5q31.1	chr5:3	5:133848917	rs10900829	*BC032795,LINC01843*	4.4 e -14	1
5p13.2	chr5:4	5:36646194	rs3776573	*SLC1A3*	1.4 e -11	1
5q31.3	chr5:5	5:139637565	rs13161154	*PFDN1,CYSTM1*	1.1 e -10	1
5q33.3	chr5:6	5:158244083	rs1432679	*EBF1*	1.2 e -9	2
5q31.1	chr5:7	5:131564501	rs111547866	*P4HA2*	1.57 e -9	1
5q31.3	chr5:8	5:141713195	rs62383568	*LOC10192641*	1.59 e -9	1
Chromosome 6: 34 independent primary signals clustered in 19 loci						
6p21.31	**chr6:1**	**6:33715159**	**rs60447213**	***IP6K3***	**3.9 e -437**	**8**
6q23.2	chr6:2	6:132049657	rs453639	*ENPP3*	3.0 e -119	5
6q23.3	chr6:3	6:136104310	rs4895457	*LINC00271,PDE7B*	6.7 e -32	2
6p21.1	chr6:4	6:44647753	rs56078331	*SUPT3H*	1.0 e -31	1
6q21	chr6:5	6:108944165	rs3813498	*FOXO3*	5.9 e -27	1
6q22.33	chr6:6	6:127144683	rs13194508	*AK127472*	6.7 e -20	1
6q23.2	chr6:7	6:134517687	rs12212449	*SGK1*	3.0 e -18	1
6q25.1	chr6:8	6:152008982	rs2941741	*ESR1*	1.8 e -17	3
6p21.33	chr6:9	6:31630665	rs59345959	*GPANK1*	1.1 e -13	2
6q13	chr6:10	6:74458737	rs9447004	*CD109*	1.3 e -13	1
6p21.31	chr6:11	6:34616322	rs3800461	*C6orf106*	1.7 e -11	1
6q23.3	chr6:12	6:137259725	rs12191772	*SLC35D3,PEX7,NHEG1*	1.8 e -11	1
6p22.2	chr6:13	6:25715657	rs116009877	*SCGN,SLC17A4*	1.0 e -10	1
6q22.31	chr6:14	6:121787498	rs9375033	*GJA1*	3.9 e -10	1
6q13	chr6:15	6:72205044	rs558092	*LINC00472,RIMS1*	7.4 e -10	1
6q26	chr6:16	6:163812707	rs1737333	*QK1*	1.1 e -9	1
6p12.2	chr6:17	6:52735206	rs9474335	*GSTA5,GSTA3*	1.3 e -9	1
6p21.1	chr6:18	6:45729130	rs17288460	*CLIC5*	2.01 e -9	1
6p22.1	chr6:19	6:27151933	rs59682554	*HIST1H2AH,PRSS16*	2.02 e -9	1
Chromosome 7: 10 independent primary signals, 10 loci						
7p21.1	chr7:1	7:20051287	rs13328356	*LOC101927668*	3.0 e -17	1
7p15.1	chr7:2	7:28479848	rs6462085	*CREBS*	3.5 e -17	1
7p21.1	chr7:3	7:17284577	rs4410790	*AHR*	6.9 e -13	1
7q21.3	chr7:4	7:97929081	rs13232861	*BAIAP2L1*	2.0 e -11	1

7q11.21	chr7:5	7:65491123	rs2460421	*ASL,GUSB,CRCP*	4.8 e -11	1
7p14.1	chr7:6	7:38144898	rs6963134	*STARD3NL,EPDR1*	5.4 e -11	1
7p22.1	chr7:7	7:6104265	rs112367067	*USP42,EIF2AK1*	2.8 e -11	1
7q22.1	chr7:8	7:101939127	rs2242581	*SH2B2*	8.3 e -10	1
7p22.3	chr7:9	7:299850	rs36170987	*FAM20C*	1.7 e -9	1
7q36.1	chr7:10	7:151414329	rs10265221	*PRKAG2*	2.8 e -9	1
Chromosome 8: 6 independent primary signals clustered in 5 loci						
8q13.3	chr8:1	8:72507296	rs6983239	*EYA1,LOC100132641*	6.2 e -20	1
8q21.13	chr8:2	8:82670771	rs35094336	*CHMP4C*	5.8 e -16	1
8p23.1	chr8:3	8:8198306	rs4840337	*SGK223,PRAG1*	1.2 e -14	1
8q21.11	chr8:4	8:76478616	rs2941483	*HNF4G*	1.5 e -13	1
8q24.21	chr8:5	8:129950004	rs13264707	*LINC00824,MIR1208*	8.7 e -12	2
Chromosome 9: 10 independent primary signals clustered in 9 loci						
9q21.11	chr9:1	9:71461815	rs10869403	*PIP5K1B*	2.2 e -21	2
9q34.3	chr9:2	9:140130606	rs28542318	*SLC34A3*	3.7 e -12	1
9q33.3	chr9:3	9:129294976	rs10819178	*MVB12B*	4.1 e -12	1
9q21.13	chr9:4	9:77499796	rs11144134	*TRPM6*	8.3 e -12	1
9q21.2	chr9:5	9:80702276	rs4877397	*CEP78,GNAQ*	1.1 e -10	1
9p24.3	chr9:6	9:908606	rs1033831	*DMRT1*	3.3 e -10	1
9p23	chr9:7	9:14020436	rs10115804	*NF1B*	8.8 e -10	1
9q34.11	chr9:8	9:130681892	rs4837205	*ST6GALNAC4,PIP5KL1*	2.4 e -9	1
9p24.1	chr9:9	9:4746539	rs447124	*AK3,RCL1*	3.2 e -9	1
Chromosome 10: 13 independent primary signals clustered in 11 loci						
10p14	chr10:1	10:9321916	rs111817798	*LOC10192672,LINC00709*	1.1 e -31	2
10q11.23	chr10:2	10:50313621	rs4509682	*VSTM4*	5.2 e -23	1
10p12.31	chr10:3	10:22280664	rs6650130	*DNAJC1*	9.1 e -18	1
10q26.3	chr10:4	10:130919942	rs10764855	*MGMT, MK167*	2.2 e -17	1
10q25.3	chr10:5	10:116080764	rs758211	*AFAP1L2*	6.7 e -17	1
10q24.2	chr10:6	10:101829514	rs61751507	*CPN1*	5.8 e -15	1
10q26.13	chr10:7	10:126419384	rs10901812	*FAM53B*	1.0 e -14	1
10p15.1	chr10:8	10:5254188	rs7897431	*AKR1C4*	9.0 e -14	1
10p14	chr10:9	10:8090380	rs1976684	*GATA3-AS1*	1.8 e -12	1
10q24.33	chr10:10	10:105057561	rs11191643	*PCGF6,INA*	2.3 e -10	2
10q22.3	chr10:11	10:80202907	rs2692723	*LINC00856*	3.1 e -9	1
Chromosome 11: 9 independent primary signals, 9 loci						
11q13.1	chr11:1	11:65337251	rs1346	*SSSCA1-AS1*	3.1 e -23	1
11p15.3	chr11:2	11:11436233	rs56002986	*GALNT18*	2.8 e -18	1
11p15.2	chr11:3	11:13550209	rs10832053	*PTH*	3.7 e -16	1
11p14.1	chr11:4	11:30806998	rs2021807	*DCDC5*	2.3 e -14	1
11q24.2	chr11:5	11:126218773	rs17135401	*ST3GAL4-AS1*	5.2 e -12	1

11q14.2	chr11:6	11:87901236	rs302650	*RAB38*	4.7 e -11	1
11q21	chr11:7	11:95223017	rs4753680	*FAM76B,CEP57,SESN3*	2.0 e -10	1
11p13	chr11:8	11:33815975	rs11601836	*FBXO3*	4.27 e -9	1
11p15.4	chr11:9	11:5573701	rs2647602	*HBG2*	4.29 e -9	1
Chromosome 12: 28 independent primary signals clustered in 14 loci						
12p13.32	**chr12:1**	**12:4606168**	**rs2970818**	***C12orf4,FGF23,FGF6***	**5.7 e -214**	**11**
12p13.2	chr12:2	12:12167729	rs4763751	*ETV6*	8.2 e -50	2
12q23.3	chr12:3	12:107297461	rs6539285	*C12orf23,TMEM26,RIC8B*	1.1 e -46	1
12q24.12	chr12:4	12:111884608	rs3184504	*SH2B3*	1.7 e -27	1
12q22	chr12:5	12:94311934	rs74737968	*LOC105369911*	3.0 e -27	3
12q24.33	chr12:6	12:131582952	rs12579593	*GPR133*	2.9 e -25	1
12q23.3	chr12:7	12:108996922	rs34826644	*TMEM119,SELPLG*	3.7 e -23	1
12q24.31	chr12:8	12:121416864	rs1800574	*HNF1A*	1.8 e -16	1
12q24.31	chr12:9	12:123560731	rs596940	*PITPNM2*	2.3 e -15	2
12q13.3	chr12:10	12:56860020	rs2939302	*MIP*	4.6 e -12	1
12q24.31	chr12:11	12:122630285	rs3809117	*MLXIP*	4.0 e -11	1
12q24.13	chr12:12	12:113563105	rs35026779	*RASAL1*	5.8 e -10	1
12q14.1	chr12:13	12:58135420	rs12371356	*AGAP2*	9.3 e -10	1
12p12.2	chr12:14	12:21254963	rs4762813	*SLCO1B1,SLCO1B7*	9.5 e -10	1
Chromosome 13: 3 independent primary signals, 3 loci						
13q13.1	chr13:1	13:33509030	rs7324259	*LINC00423*	1.2 e -13	1
13q31.1	chr13:2	13:80749431	rs9574586	*SPRY2*	1.1 e -9	1
13q14.3	chr13:3	13:51385422	rs1570603	*DLEU7,DLEU7-AS1*	3.3 e -9	1
Chromosome 14: 2 independent primary signals, 2 loci						
14q32.11	chr14:1	14:91449394	rs1286070	*RPS6KA5*	8.0 e -17	1
14q11.2	chr14:2	14:23452128	rs3811183	*AJUBA,C14orf93*	2.7 e -9	1
Chromosome 15: 4 independent primary signals clustered in 3 loci						
15q21.2	chr15:1	15:51104350	rs12909505	*AK091906,SPPL2A*	2.2 e -37	1
15q24.1	chr15:2	15:75079474	rs34933034	*CSK*	1.1 e -18	2
15q22.2	chr15:3	15:60883281	rs339969	*RORA*	2.7 e -13	1
Chromosome 16: 9 independent primary signals clustered in 8 loci						
16p13.11	chr16:1	16:16259596	rs41278174	*ABCC6*	3.5 e -53	1
16q23.2	chr16:2	16:79755446	rs4575545	*MAFTRR*	5.9 e -47	2
16p13.3	chr16:3	16:4016676	rs2230742	*ADCY9*	6.1 e -18	1
16q24.2	chr16:4	16:88516171	rs4782365	*ZFPM1*	3.5 e -13	1
16q12.1	chr16:5	16:49888931	rs9745989	*ZNF423*	1.8 e -12	1
16q22.3	chr16:6	16:73024276	rs1858800	*ZFHX3*	4.3 e -11	1
16q23.2	chr16:7	16:81534790	rs2925979	*CMIP*	1.0 e -9	1
16q22.2	chr16:8	16:72147983	rs112953704	*DHX38*	4.0 e -9	1

Chromosome 17: 18 independent primary signals clustered in 15 loci						
17q24.2	chr17:1	17:66703728	rs11871728	*LINC01482,ABCA8*	2.6 e -65	2
17q12	chr17:2	17:37504933	rs8069451	*FBXL20*	3.5 e -39	1
17p13.3	chr17:3	17:1618363	rs11078597	*MIR22HG, ABR*	1.6 e -35	2
17q23.2	chr17:4	17:58996208	rs2120222	*BCAS3*	3.0 e -34	2
17q21.31	chr17:5	17:44297148	rs2696689	*KANSL1*	8.0 e -28	1
17q25.3	chr17:6	17:76933645	rs7221260	*TIMP2, LGALS3BP*	2.1 e -19	1
17p11.2	chr17:7	17:17947710	rs2955382	*GID4*	3.5 e -17	1
17q21.32	chr17:8	17:45765249	rs6503796	*KPNB1,TBKBP1*	3.8 e -16	1
17q23.1	chr17:9	17:57790627	rs9903626	*VPM1*	1.5 e -13	1
17p13.1	chr17:10	17:10031823	rs28391220	*GAS7*	7.0 e -13	1
17q11.2	chr17:11	17:29726603	rs4795607	*RAB11FIP4*	1.0 e -12	1
17p13.1	chr17:12	17:7213579	rs73977632	*EIF5A*	1.9 e -10	1
17q21.31	chr17:13	17:42090587	rs228778	*TMEM101*	3.4 e -10	1
17q21.32	chr17:14	17:47022755	rs4794000	*UBE2Z,SNF8,GIP*	6.2 e -10	1
17q22	chr17:15	17:53361838	rs1477141	*HLF*	7.3 e -10	1
Chromosome 19: 10 independent primary signals clustered in 9 loci						
19p13.11	chr19:1	19:17504801	rs34466478	*BSJ2,AK311380*	1.0 e -25	1
19p13.11	chr19:2	19:18575193	rs78030362	*ELL*	1.1 e -19	1
19p13.3	chr19:3	19:3102748	rs308032	*GNA11*	2.3 e -16	1
19q13.2	chr19:4	19:41839631	rs8105161	*TGFB1*	2.2 e -12	1
19q13.41	chr19:5	19:53405497	rs10401230	*ZNF888,ZNF320*	5.6 e -11	1
19q13.12	chr19:6	19:35554479	rs28616221	*HPN*	1.3 e -10	2
19p13.12	chr19:7	19:14172921	rs10415758	*PALM3,MISP3,LOC113230*	4.2 e -10	1
19q13.32	chr19:8	19:46385540	rs34863090	*IRF2BP1*	2.0 e -9	1
19q13.2	chr19:9	19:38817628	rs35496032	*KCNK6*	2.07 e -9	1
Chromosome 20: 8 independent primary signals clustered in 5 loci						
20q12	chr20:1	20:39344272	rs3091842	*MAFB,TOP1*	4.9 e -30	3
20q13.2	chr20:2	20:52732362	rs17216707	*BCAS1,CYP24A1*	1.7 e -24	2
20q13.12	chr20:3	20:45569796	rs6124901	*EYA2*	3.3 e -10	1
20q13.13	chr20:4	20:48981014	rs6063504	*LOC264751,LINC01270*	1.0 e -9	1
20q13.12	chr20:5	20:43042364	rs1800961	*HNF4A*	4.4 e -9	1
Chromosome 22: 2 independent primary signals, 2 loci						
22q13.1	chr22:1	22:38599857	rs140916674	*MAFF*	1.9 e -19	1
22q11.22	chr22:2	22:23365501	rs6003465	*RSPH14,RTDR1*	1.1 e -9	1
Chromosome X, men: 3 independent primary signals clustered in 2 loci						
Xp22.12	chrXm:1	X:22039936	rs178676	*PHEX*	2.0 e -66	2
Xp22.31	chrXm:2	X:8902853	rs5934503	*FAM9A, FAM9B*	4.1 e -14	1
Chromosome X, women: 1 independent primary signal						
Xp22.11	chrXw:1	X:22043385	rs178687	*PHEX, SMS*	4.4 e -43	1

Chromosomal band and positioning was established according to GRCh 37 assembly. Sentinel SNP: independent SNP with lowest p value in locus. Lowest p: lowest *p* value in locus. # SNPs: number of independent SNPs associated with P levels per locus. The three top hit SNPs are highlighted in bold. ChrXm: locus in X-chromosome in men; chrXw: locus in X-chromosome in women.

Suppl. Table 2. SNPs with suggestive *p* value for sex difference in UKBB participants with European ancestry

SNP	rsID	EA	*β* men	*p* men	*β* women	*p* women	*p* sex difference
1:90813258	rs78025300	C	0.78	0.00026	-1.01	0.00003	5.1 e -9
5:110419326	rs150431495	G	0.026	0.023	-0.05	8.1 e -7	1.4 e -8
21:24005210	rs549697724	A	0.085	1.5 e -6	-0.039	0.0079	2.2 e -8

Suppl. Table 3a. Summary of results from fine-mapping approach in the first top hit locus (6p21.31) in UKBB participants with European ancestry

Chr	Pos	rsID	P. Probability
6	33713930	rs79297236	0.000001
6	33683068	rs77528762	0.00001
6	33692368	rs7744811	0.00001
6	33667660	rs73743305	0.00004
6	33689299	rs73743310	0.00013
6	33705355	rs73743323	0.00022
6	33649461	rs73743304	0.00031
6	33683548	rs73743309	0.00051
6	33700004	rs73743322	0.00058
6	33710284	rs6942022	0.00075
6	33715391	rs73743336	0.00075
6	33709980	rs73743326	0.00077
6	33714859	rs16871391	0.00082
6	33715265	rs16869466	0.00089
6	33714908	rs16869459	0.00109
6	33714622	rs16869456	0.00110
6	33713971	rs73743330	0.00119
6	33713550	rs73743328	0.00131
6	33715127	rs16869464	0.00149
6	33706479	rs9469578	0.00186
6	33706159	rs57303110	0.00221
6	33707976	rs6929527	0.00261

6	33714412	rs78748610	0.00330
6	33715837	rs9469580	0.00330
6	33715904	rs9469581	0.00352
6	33707112	rs6923565	0.36208
6	33714042	rs73743333	0.36386
6	33704601	rs59957785	0.36751
6	33714036	rs73743331	0.37337
6	33714639	rs16869458	0.61789
6	33715159	rs60447213	0.61793
6	33715939	rs9469582	0.62963
6	33716945	rs73743401	0.63092
6	33685417		0.99259
6	33658780	rs35506178	0.99997

P. probability: posterior probability of causality

Results displayed in ascending order of probability of causality

Only genotyped SNPs or imputed SNPs with INFO>0.9 were included

Suppl. Table 3b. Summary of results from fine-mapping approach in the second top hit locus (12p13.32) in UKBB participants with European ancestry

Chr	Pos	rsID	P. Probability
12	4570397	rs7133907	7.97 e -69
12	4688634	rs10492054	1.8 e -66
12	4578609	rs10849080	3.11 e -64
12	4481044	rs13312789	1.68 e -62
12	4555754	rs2970822	1.1 e -61
12	4479549	rs7955866	2.98 e -61
12	4495864	rs12815443	9.60 e -57
12	4675548	rs7973864	1.2 e -57
12	4500711	rs12820100	2.3 e -55
12	4554413	rs11611403	4.06 e -55
12	4486682	rs11063121	8.83 e -53
12	4484459	rs7976548	4.33 e-51
12	4505588	rs2358955	2.91 e -51
12	4570190	rs7133648	1.92 e -49
12	4623381	rs11503123	8.23 e -48

12	4576289	rs2358958	9.93 e -48
12	4498407	rs10849051	2.24 e -47
12	4650782	rs11063217	1.10 e -46
12	4565117	rs2909372	5.51 e -46
12	4498015	rs11063129	2.19 e -45
12	4506008	rs2098509	4.43 e -45
12	4556636	rs3812823	2.15 e -43
12	4667282	rs11063227	7.43 e -44
12	4554630	rs11613495	9.90 e -44
12	4651494	rs11063220	5.21 e -43
12	4562344	rs6489546	2.10 e -42
12	4568745	12:4568745_AAT_A	2.10 e -42
12	4495087	rs16931344	2.68 e -42
12	4493770	rs7316649	4.60 e -42
12	4492315	rs2075317	1.44 e -41
12	4486893	12:4486893_GC_G	1.66 e -39
12	4588579	rs11063191	6.11 e -39
12	4566423	rs11833654	1.16 e -38
12	4563810	rs7315434	3.00 e -38
12	4569663	rs140034971	3.00 e -38
12	4565436	12:4565436_TA_T	1.36 e -36
12	4566067	rs10849071	1.36 e -36
12	4497672	rs7308018	1.61 e -36
12	4489235	rs3812822	5.65 e -36
12	4675458	rs7973740	6.49 e -36
12	4495795	rs10744645	6.50 e -36
12	4644879	rs10849085	1.56 e -35
12	4568392	rs12320102	2.06 e -35
12	4591336	rs11063197	4.25 e -35
12	4603698	rs11063207	5.90 e -34
12	4541155	rs11613677	8.75 e -34
12	4591262	rs11063195	1.08 e -33
12	4636814	rs11063215	1.74 e -33
12	4637078	rs200953533	2.28 e -33
12	4486753	rs7966472	2.47 e -33
12	4486797	rs10849050	3.40 e -33
12	4569117	rs11063161	3.97 e -33
12	4579106	rs11063177	2.69 e -32

12	4485695	rs140174060	1.40 e -31
12	4575269	rs10849077	4.69 e -31
12	4662804	12:4662804_CT_C	4.69 e -31
12	4486618	rs11063120	4.70 e -31
12	4661387	rs10849089	9.44 e -30
12	4616901	rs2907499	1.28 e -29
12	4567922	rs7959292	1.29 e -29
12	4672096	rs7295624	1.29 e -29
12	4636520	rs534409387	2.65 e -29
12	4492806	rs2075316	3.06 e -29
12	4573791	rs11063169	4.01 e -29
12	4487865	rs11063123	2.93 e -28
12	4601068	rs9971728	2.93 e -28
12	4583182	rs11063183	5.63 e -28
12	4667910	rs7308641	8.84 e -28
12	4573252	rs73037722	9.57 e -28
12	4670931	rs7304989	9.57 e -28
12	4650065	rs10849087	1.54 e -27
12	4602721	rs63035560	1.83 e -27
12	4486786	rs11063122	1.02 e -26
12	4669740	rs7965755	3.48 e -26
12	4623515	rs11063211	3.83 e -26
12	4671490	rs740059	1.03 e -25
12	4642184	rs2907494	1.32 e -25
12	4591854	rs11063199	2.13 e -25
12	4638164	rs2907495	2.13 e -25
12	4642572	rs10849083	2.00 e -24
12	4658749	rs7972086	2.00 e -24
12	4562946	rs10774235	2.00 e -24
12	4670382	rs10849091	2.55 e -24
12	4608325	rs11063208	1.88 e -23
12	4576961	rs11063172	4.56 e -23
12	4591351	rs11063198	3.77 e -22
12	4638102	12:4638102_CA-CAA_C	3.77 e -22
12	4671612	rs7305896	6.63 e -22
12	4568713	rs61909577	2.37 e -21
12	4587910	rs11063187	2.37 e -21

12	4660259	rs12314323	4.09 e -21
12	4481371	rs11063116	6.05 e -21
12	4588248	rs11063189	6.05 e -21
12	4592341	rs11063200	6.05 e -21
12	4608103	rs2970810	6.05 e -21
12	4672394	rs7299185	6.05 e -21
12	4590963	rs552322663	8.42 e -21
12	4581950	rs7965800	9.47 e -21
12	4628899	rs2907498	9.47 e -21
12	4640362	rs2970808	9.97 e -21
12	4609625	rs7304949	1.20 e -20
12	4563440	rs10431358	1.55 e -20
12	4645400	rs10849086	1.61 e -20
12	4588230	rs11063188	1.77 e -20
12	4597481	rs10849081	1.77 e -20
12	4616642	rs4238020	1.77 e -20
12	4682626	rs61909600	1.77 e -20
12	4591100	rs11063193	5.01 e -20
12	4671926	rs7298545	5.01 e -20
12	4595291	rs61909580	5.02 e -20
12	4486888	rs61909253	7.79 e -20
12	4495732	rs11063127	1.28 e -18
12	4625836	rs2970812	1.28 e -18
12	4555575	rs2970823	3.24 e -18
12	4622781	rs61909595	3.24 e -18
12	4637864	rs73037770	3.25 e -18
12	4500608	rs17773299	4.77 e -18
12	4566259	rs10774237	4.77 e -18
12	4597745	rs11063205	4.77 e -18
12	4507719	rs11063133	1.07 e -17
12	4591244	rs11063194	1.07 e -17
12	4645448	rs1029769	1.07 e -17
12	4566866	rs12317924	1.07 e -17
12	4580488	rs11063179	1.50 e -17
12	4570008	rs28801989	1.50 e -17
12	4563894	rs10431359	1.55 e -17
12	4607342	rs2909380	1.55 e -17
12	4507462	rs2909370	2.96 e -17

12	4581371	rs11063182	2.96 e -17
12	4606527	rs2909381	3.09 e -17
12	4502410	rs7967051	4.03 e -17
12	4497214	12:4497214_GT_G	1.24 e -16
12	4569640	rs28836512	1.24 e -16
12	4577468	rs10849079	1.24 e -16
12	4672019	rs10689238	1.24 e -16
12	4602996	rs11063206	3.86 e -16
12	4635045	rs11063214	3.86 e -16
12	4506123	rs11063132	1.50 e -15
12	4615481	rs4323977	1.50 e -15
12	4675126	rs7957926	1.63 e -15
12	4486886	rs61909252	1.63 e -15
12	4580657	rs11063181	1.78 e -15
12	4603123	rs9971700	6.81 e -14
12	4635913	rs12367035	6.81 e -14
12	4636095	rs12366731	6.81 e -14
12	4593690	rs11063202	6.81 e -14
12	4575084	rs11063170	6.83 e -14
12	4566457	rs11836032	7.00 e -14
12	4562320	rs11063158	9.17 e -14
12	4657203	rs7314915	9.17 e -14
12	4669954	rs10849090	9.17 e -14
12	4486722	rs7978281	9.17 e -14
12	4585428	rs7968405	9.17 e -14
12	4486891	rs61909254	1.60 e -13
12	4493938	rs720333	1
12	4562881	rs10774234	1
12	4596782	rs71579224	1
12	4632967	rs11063213	1

P. probability: posterior probability of causality

Results displayed in ascending order of probability of causality

Only genotyped SNPs or imputed SNPs with INFO>0.9 were included

Suppl. Table 3c. Summary of results from fine-mapping approach in the third top hit locus (1p36.12) in UKBB participants with European ancestry

Chr	Pos	rsID	P. Probability
1	21976938	rs3767118	8.52 e -137
1	21844590	rs113324018	1.15 e -125
1	21843256	rs12116570	1.03 e -124
1	21736898	rs12726147	1.23 e -123
1	21843498	rs28508264	4.03 e -123
1	21304003	rs78430217	3.41 e -122
1	21841697	rs972662	1.71 e -121
1	21826566	rs6667242	8.25 e -120
1	21842461	rs72874247	8.99 e -128
1	21842892	rs12116444	1.60 e -117
1	21844496	rs80320018	1.66 e -117
1	21626931	rs12758257	3.52 e -117
1	21632187	rs12747397	1.70 e -114
1	21888425	rs141276685	3.14 e -113
1	21684950	rs149118844	2.81 e -111
1	21741338	rs36103528	2.65 e -110
1	22046558	rs10799708	3.09 e -110
1	21830686	rs13353029	2.17 e -109
1	21801346	rs7531217	2.17 e -109
1	21777426	rs4654747	4.22 e -108
1	21765211	rs6680628	7.67 e -108
1	21844638	rs113393066	1.12 e -107
1	21227031	rs72652959	2.84 e -107
1	21776987	rs58431050	1.56 e -106
1	21386013	rs781308545	1.88 e -106
1	21701057	rs182314176	1.97 e -105
1	21829452	rs1936823	9.90 e -105
1	21803664	rs7527656	1.87 e -104
1	21802453	rs6669637	9.29 e -104
1	21787731	rs4654939	2.41 e -103
1	21842665	rs111330128	4.00 e -103
1	21800185	rs7520015	4.49 e -103
1	21916749	rs113298409	9.32 e -103
1	22005665	rs1059218	1.51 e -102

1	21040310	rs72650882	2.82 e -101
1	21961797	rs60718161	2.13 e -100
1	21786750	rs12738545	2.42 e -100
1	21836340	rs10917002	3.61 e -99
1	21821005	rs79541959	4.73 e -99
1	21775805	1:21775805_GA_G	2.94 e -98
1	21833925	rs12066162	5.16 e -97
1	21622013	rs1067237	6.65 e -97
1	21821117	rs61778366	1.16 e -96
1	21795824	rs4654752	2.94 e -96
1	21813617	rs10799696	3.36 e -96
1	21496711	rs114987014	7.59 e -96
1	21787745	rs141472552	1.19 e -95
1	21810757	rs143325351	4.16 e -95
1	21915110	rs11582381	6.69 e -95
1	21801116	rs7554122	3.42 e -94
1	21827179	rs55805990	1.62 e -93
1	21842687	rs72874253	1.86 e -93
1	21761309	rs4079441	3.66 e -93
1	21963740	rs115589123	1.03 e -92
1	21927809	1:21927809_TC_T	1.23 e -92
1	21171386	rs72652923	1.26 e -92
1	21814187	rs12566538	1.59 e -92
1	21836934	rs6703976	1.67 e -92
1	21845550	rs10917003	5.41 e -92
1	21802029	rs4654945	1.25 e -91
1	21915855	rs72660304	1.79 e -91
1	21738259	rs35836191	2.14 e -91
1	21682194	rs10916966	2.96 e -91
1	21789442	rs6660003	2.97 e -91
1	21785981	rs4654938	7.61 e -91
1	21843401	rs12119205	1.04 e -90
1	21839690	rs975000	1.55 e -90
1	21679459	1:21679459_AAATT_A	2.36 e -90
1	21830340	rs56702468	3.00 e -90
1	21685036	rs183934675	4.28 e -90
1	21847548	rs12145027	6.38 e -90
1	21833509	rs72659158	7.51 e -90

1	21790941	rs6426709	9.73 e -90
1	21759839	rs12759170	1.04 e -89
1	21643162	rs72654902	1.37 e -89
1	21827094	rs56155961	1.91 e -89
1	21813143	rs2047653	6.88 e -89
1	21660361	rs61781598	3.01 e -88
1	21828619	rs12566622	3.50 e -88
1	21495668	rs115842520	3.50 e -88
1	21961337	rs78513539	8.28 e -88
1	21775849	rs112985962	1.30 e -87
1	21798866	rs7514120	1.30 e -87
1	21125324	rs149626316	1.39 e -87
1	21701970	rs201574317	3.20 e -87
1	21839730	rs869180	9,53 e -87
1	21831494	rs34828194	1.39 e -86
1	21796616	rs7537914	5.26 e -86
1	21777751	rs4311854	1.26 e -85
1	21630280	rs12723450	7.13 e -85
1	21944397	rs1318235	8.81 e -85
1	21698405	rs72657133	1.66 e -84
1	21513911	rs72654867	2.33 e -84
1	21197471	rs72652944	2.38 e -84
1	21935467	rs12130950	3.17 e -84
1	21975696	rs74671214	5.49 e -84
1	21926498	rs1767444	6.52 e -84
1	21680310	rs3026793	6.61 e -84
1	22109952	rs41265999	8.42 e -84
1	21821897	rs1697422	9.61 e -84
1	21844736	rs12022032	3.99 e -83
1	21909186	rs12141777	9.76 e -83
1	21831163	rs7520712	1.07 e -82
1	21210784	rs74998260	1.28 e -82
1	21792752	rs3968525	1.34 e -82
1	21728347	rs533408226	1.50 e -82
1	21876957	rs116121521	1.65 e -82
1	21805730	rs2316504	2.69 e -82
1	21934693	rs72660316	3.64 e -82
1	21809524	rs2800941	3.98e -82

1	21800219	rs10916996	5.64 e -82
1	21843991	rs113269463	1.50 e -81
1	21780651	rs2010202	4.28 e -81
1	21852781	rs6658446	9.91 e -81
1	21821880	rs10799702	1.14 e -80
1	22070679	rs140816439	1.17 e -80
1	21807880	1:21807880_CCTCTGTCT_C	5.45 e -80
1	21799823	rs1971328	1.21 e -79
1	21840048	rs61778369	1.21 e -79
1	21947216	rs11588121	9.48 e -79
1	21832624	rs142284739	1.09 e -78
1	21785287	rs6666997	2.04 e -78
1	21775497	rs61775934	2.66 e -78
1	21863133	rs1697411	2.66 e -78
1	21683948	rs116236981	3.97 e -78
1	21659826	rs147254696	4.35 e -78
1	21812915	1:21812915_CTGGA_C	9.47 e -78
1	21890386	rs1256335	9.47 e -78
1	21844875	rs72874261	8.25 e -77
1	21682110	rs10916964	8.27 e -77
1	21683947	rs115937364	1.07 e -76
1	21852819	rs6704518	1.69 e -76
1	21806821	rs2800935	1.81 e -76
1	21661691	rs61781599	5.77 e -76
1	21808674	rs115166616	7.77 e -76
1	21911345	rs144208700	7.77 e -76
1	21854503	rs12027423	7.78 e -76
1	21794061	rs17420195	9.10 e-76
1	21516090	rs34554534	1.67 e -75
1	21847111	rs138293109	1.97 e -75
1	21787018	rs2316509	2.30 e -75
1	22001971	rs829395	3.80 e -75
1	21665405	rs61781600	6.84 e -75
1	21812252	rs71512998	7.32 e -75
1	21935053	rs115070771	9.26 e -75
1	21803734	rs7527672	1.03 e -74
1	21919370	rs72660309	2.70 e -74
1	21807864	rs78885464	4.53 e -74

1	21684697	rs72657121	4.60 e -74
1	21814668	rs34061014	6.26 e -74
1	21775834	rs150401191	6.33 e -74
1	21786421	rs538218624	6.33 e -74
1	21961138	rs116680485	6.56 e -74
1	21759020	rs10916988	7.66 e -74
1	21842530	rs72874249	1.86 e -73
1	21797419	rs4654944	1.95 e -73
1	21842558	rs1697399	1.45 e -72
1	21685431	rs7530820	2.69 e -72
1	21844769	rs72874260	7.53 e -72
1	21969922	rs7531180	1.22 e -71
1	21681754	1:21681754_ATTTG_A	1.42 e -71
1	21865230	rs1697412	2.47 e -71
1	21814260	rs12565696	2.77 e -71
1	21792753	rs3968524	3.44 e -71
1	21829768	rs149740088	7.36 e -71
1	21680408	rs3026792	3.40 e -70
1	21980681	rs3767126	3.92 e -70
1	21778035	rs4654937	4.65 e -70
1	21682361	rs10916967	2.24 e -69
1	21824108	rs1780321	2.24 e -69
1	21634612	rs35696560	4.19 e -69
1	21684087	rs10916968	1.02 e -68
1	21879568	rs58060846	1.12 e -68
1	21689382	rs531429510	1.81 e -68
1	21795388	rs1827293	1.81 e -68
1	21832730	rs3856178	2.82 e -68
1	21936835	rs116251894	7.34 e -68
1	21775943	rs10799692	1.76 e -67
1	21800703	rs11453851	1.22 e -66
1	21863384	1:21863384_CTTT_C	1.81 e -66
1	21805978	rs4654948	1.88 e -66
1	21817365	rs75824579	1.88 e -66
1	21681267	rs12082897	1.88 e -66
1	21786938	rs12563420	9.01 e -66
1	21814390	rs6680125	1.04 e -65
1	21716182	rs369124092	1.16 e -65

1	21830659	rs13353032	1.23 e -65
1	21824641	rs202247715	1.54 e -65
1	21833856	rs12092961	1.67 e -65
1	21895625	rs1780318	1.79 e -65
1	21963579	rs35272791	3.31 e -65
1	21681924	rs10916963	7.99 e -65
1	21796770	rs6687836	7.99 e -65
1	21802122	rs4654946	8.28 e -65
1	21988563	rs829373	2.49 e -64
1	21786423	rs569570049	1.15 e -63
1	21683808	rs12059998	1.37 e -63
1	21809436	rs199855186	3.47 e -63
1	21796517	rs10632199	8.88 e -63
1	21805048	rs4654947	1.10 e -62
1	21777452	rs4654936	2.42 e -62
1	21820990	rs10799701	2.42 e -62
1	21989979	rs853479	2.42 e -62
1	21671973	rs114921587	4.95 e -62
1	21817397	rs72659148	5.64 e -62
1	21267125	rs72652978	1.76 e -61
1	21916829	rs1256280	1.76 e -61
1	21856119	rs1697407	4.46 e -61
1	21778965	rs2016464	4.46 e -61
1	21702291	rs12094288	1.43 e -60
1	21865495	rs4654961	1.43 e -60
1	21950828	rs72660334	1.90 e -60
1	21765690	rs61775933	1.90 e -60
1	21952884	rs1130564	2.57 e -60
1	21795432	rs35175190	6.25 e -60
1	21728343	rs144972831	6.59 e -60
1	21776650	rs35384882	6.59 e -60
1	21829615	rs1936824	6.59 e -60
1	21346175	rs145791784	3.42 e -59
1	21775851	rs182212109	3.42 e -59
1	21766605	rs536981426	7.37 e -59
1	21680008	rs72657116	8.29 e -59
1	21939036	rs11586663	8.43 e -59
1	21803177	rs6426713	1.80 e -58

1	21916738	rs112111282	4.29 e -58
1	21776392	rs10737457	3.07 e -57
1	21823629	rs1697420	4.93 e -57
1	22003793	rs829396	4.93 e -57
1	21691933	rs6682015	1.30 e -56
1	21806447	rs3820296	1.34 e -56
1	21775951	rs145038894	1.35 e -56
1	21710044	rs72657146	2.4 e -56
1	21817085	rs1106357	2.42 e -56
1	21965163	rs6665848	2.91 e -56
1	21630291	rs12737859	2.92 e -56
1	21968741	rs78915636	2.92 e -56
1	21879643	rs57915975	2.92 e -56
1	21806874	rs56414407	4.61 e -56
1	21733014	rs72657176	7.83 e -56
1	21958587	rs10799705	1.35 e -55
1	21977332	rs78356267	1.35 e -55
1	21683553	rs200864008	1.19 e -54
1	21780834	rs10737459	1.19 e -54
1	21776121	rs10737456	4.70 e -54
1	21785229	rs6675223	3.49 e -53
1	21786422	rs550139712	5.19 e -53
1	21802450	rs6657015	5.19 e -53
1	21759128	rs10916989	6.21 e -53
1	21817126	rs80212518	6.21 e -53
1	21823076	rs1780322	6.21 e -53
1	21739882	rs61781636	1.32 e -51
1	21786068	rs4654748	1.32 e -51
1	21981361	rs4654763	1.32 e -51
1	21799489	rs1971327	5.95 e -51
1	21680578	rs12095581	7.11 e -51
1	21755772	rs61775924	7.11 e -51
1	21933588	rs72660315	7.11 e -51
1	21980325	rs3767124	7.11 e -51
1	21987094	rs4654976	2.52 e -50
1	21435580	rs138695205	3.43 e -50
1	21694417	rs6665817	3.43 e -50
1	21933568	rs72660314	3.52 e -49

1	21779465	rs2016413	3.52 e -49
1	21837755	rs16825455	3.52 e -49
1	21706420	rs72657142	4.26 e -49
1	21820961	rs10799699	4.26 e -49
1	21771344	rs3820597	1.76 e -48
1	21956759	rs199787255	1.88 e -48
1	21690632	rs72657127	2.32 e -48
1	21731734	rs7555660	2.32 e -48
1	21726849	rs116363246	1.14 e -47
1	21978648	rs75141928	1.14 e -47
1	21860427	rs771549931	2.31 e -47
1	21784093	rs4515756	3.82 e -47
1	21818297	1:21818297_CCTGT_C	3.82 e -47
1	21766453	rs1976403	1.21 e -45
1	21890264	1:21890264_TTGA_T	2.31 e -45
1	21821757	rs1780324	1.38 e -44
1	21684340	rs10916969	2.31 e -44
1	21801838	rs4654753	2.31 e -44
1	21929655	rs41307882	2.31 e -44
1	21999320	rs829401	7.58 e -43
1	21693772	rs200188919	2.09 e -41
1	21779737	rs2016203	2.09 e -41
1	21806337	rs147900768	2.09 e -41
1	21944296	rs115641764	2.09 e -41
1	21701289	rs11808970	9.86 e -41
1	21783123	1:21783123_CATTTGGCAAAATT-G_C	9.86 e -41
1	21810586	rs78156905	9.86 e -41
1	22023984	rs144527086	9.86 e -41
1	21699486	rs72657136	3.76 e -38
1	21865238	1:21865238_AGCCAGGTCTTGAA-T_A	3.76 e -38
1	21794612	rs7541779	3.80 e -38
1	21806792	rs56286426	1.45 e -37
1	21901938	rs61778392	1.45 e -37
1	21922132	rs55722102	1.45 e -37
1	21823292	rs1697421	4.80 e -37
1	21713457	rs186354921	1.04 e -35

1	21813152	rs2047654	1.04 e -35
1	21873261	rs115626047	1.04 e -35
1	21776884	rs6685071	2.15 e -34
1	21788434	rs7545635	2.15 e -34
1	21797561	rs756303105	4.39 e -34
1	21905450	rs771815228	4.39 e -34
1	21919914	rs829389	4.39 e -34
1	21780000	rs58392257	1.43 e -33
1	21850063	rs141227415	1.43 e -33
1	21863631	rs2651407	8.85 e -31
1	21684651	rs202170229	8.86 e -31
1	21554573	rs3026901	1.61 e -30
1	21806751	rs12083322	1.61 e -30
1	21854506	rs542480864	7.79 e -30
1	21909424	rs72659199	7.79 e -30
1	21783849	rs764117289	3.23 e -29
1	21947895	rs72660333	3.23 e -29
1	21819611	rs7555005	3.23 e -29
1	21797996	rs2004380	1.54 e -28
1	21916688	rs144999112	1.54 e -28
1	21905007	rs1772720	1.64 e -28
1	21985687	rs1681269	1.64 e -28
1	21767096	rs4654745	6.95 e -25
1	21914362	rs1256279	6.95 e -25
1	21927356	rs148785605	6.95 e -25
1	21930542	rs3767111	1.17 e -22
1	21965066	rs79381102	1.17 e -22
1	21900891	rs1767447	1.01 e -18
1	21679444	rs72657115	2.96 e -14
1	21770035	rs10916993	2.96 e -14
1	21819159	rs201680459	2.96 e -14
1	21829213	rs189414599	2.96 e -14
1	21934326	rs115051087	2.96 e -14
1	21989655	rs72660359	2.96 e -14
1	21994434	rs4654977	2.96 e -14
1	21713208	rs116400480	1.51 e -13
1	21988843	rs861213	1.51 e -13
1	21806619	rs3820294	1.51 e -13

1	21681868	rs143368590	1.88 e -9
1	21683950	rs114890063	1.88 e -9
1	21760127	rs12734589	1.69 e -7
1	21819373	rs7533048	1.69 e -7
1	21952024	rs1318236	1.69 e -7
1	21806926	rs2800936	1.69 e -6
1	21806624	rs3820292	1.03 e -5
1	21842922	rs10679391	1.03 e -5
1	21913619	1:21913619_CG_C	1.03 e -5
1	21993306	rs829378	1.03 e -5
1	21735016	rs72657184	4.53 e -5
1	21806621	rs3820293	4.53 e -5
1	21937559	rs72660319	4.53 e -5
1	21734938	rs72657183	0.000769791
1	21810022	rs200969812	0.000769791
1	21889635	rs1780316	0.000769791
1	21948938	rs182287953	0.000769791
1	21717027	rs72657152	0.999173
1	21944352	rs748488	0.999173
1	21959015	rs1557087	0.999173
1	21820042	rs12132412	1

P. probability: posterior probability of causality
Results displayed in ascending order of probability of causality
Only genotyped SNPs or imputed SNPs with INFO>0.9 were included

Title: The Annunciation.
Author: Fra Angelico.
Chronology: 1425-1428.
Style: Quattrocento (Renaissance)
Technique: Tempera on wood.
Location: Currently in the Prado Museum.

Título: La Anunciación.
Autor: Fra Angélico.
Cronología: 1425-1428.
Estilo: Quattrocento (Renacimiento)
Técnica: Témpera sobre tabla.
Ubicación: Actualmente en el Museo del Prado.

"In the sixth month the Angel GABRIEL was sent by GOD to a town in Galilee... to a VIRGIN betrothed to a man named JOSEPH, and the VIRGIN'S name was MARY. He went in and said to her, 'Rejoice, you who enjoy GOD's favour! The LORD is with you.'"

"Al sexto mes fue enviado por DIOS el Ángel GABRIEL a una ciudad de Galilea... a una VIRGEN desposada con un hombre llamado JOSE; el nombre de la VIRGEN era MARIA. Y entrando, le dijo: «Alégrate, llena de GRACIA, el SEÑOR está contigo.»"

[ST. LUKE (HOLY GOSPEL) 1:26-28]
[SAN LUCAS (SANTO EVANGELIO) 1:26-28]

12 Discussion

The aims of this thesis were threefold: 1) to investigate the relation of bone-related traits, such as bone mineral density (BMD) and serum phosphate (P), with several health outcomes – including all-cause and cause-specific mortality, fractures and coronary calcification; 2) to study sex differences in these associations as well as in serum calcium and phosphate levels; and 3) to discover underlying genetic variants for human serum phosphate levels and for monogenetic forms of osteoporosis. These aspects of my work will be discussed here. In addition, an outlook to future research in this area will be highlighted at the end.

Part I. Bone traits and phosphate in relation to health & survival

Bone mineral density and mortality: update in the Rotterdam Study

The Rotterdam Study has been one of the leading cohorts in reproducing an incipient and interesting finding noticed in few other research groups since 1991 and in describing that low BMD is related to mortality independently of fractures (1-3). Indeed, the knowledge of fracture events as an important factor in excess mortality was by then well established (4,5), but the novel concept of low BMD influencing survival beyond skeletal boundaries posed in itself a challenge from both the epidemiological and the biological perspectives.

It was originally proposed that this association could be suffering from confounding and that a low BMD was only reflecting a cumulative load of noxious stimuli throughout life (1,6). Despite this initial interpretation, there was a continuous growth of research articles suggesting that a low bone mass, a compromised bone microarchitecture – or a combination of both - was playing a potential role in decreasing survival in the general population (7-11). Gradually, the studies were more extensive until they reached the threshold for meta-analyses, which confirmed an epidemiological association between low BMD and all-cause mortality (12). Subsequently, the research question arose about the nature of the disease (s) underlying this relation.

In parallel to the acknowledgment of the role of BMD in survival beyond fracture-mortality, a new term arose in the literature to denote a joint condition of low BMD and arterial calcification (AC). The first description of the "calcification paradox" (13) was echoed by abundant heterogeneous reports, some showing an inverse relation between BMD and AC (especially in postmenopausal women) (14) but others found no such link (15). It became apparent that the source of heterogeneity was partly driven by sex, age, and the skeletal site (16), as lumbar spine BMD seemed to provide a more consistent evidence of the association, especially in men (17).

Despite low consistency, the relation BMD-AC acquired importance as AC constitutes an independent risk factor for CVD mortality. In a landmark report, Block et al. (18) were among the first to correctly hypothesize the ominous prognosis that AC conferred to chronic kidney disease (CKD) patients in hemodialysis. This was later replicated in CKD patients not on dialysis (19) until the evidence became so strong at the population level that a prominent position statement from the American Heart Association acknowledged the crucial role of arterial calcification in increasing cardiovascular disease and mortality, especially in the coronary bed (20).

Following this evidence and a relation between low BMD and stroke mortality (1), a possible link between BMD and CVD mortality was thoroughly investigated. However, the heterogeneity across studies precluded a confirmatory association. Indeed, the last available meta-analysis (21) describes an inverse relation between BMD and CVD mortality, but the authors highlighted the strong evidence of publication bias to the point of nullifying the results. In other words, a considerable amount of research that had not reached publication has not found BMD to be related to CVD mortality (22). Of note, the pooled evidence lacks stratified analysis according to skeletal site (12, 21), which is an important aspect as different compartments in bone can display specific properties and metabolic activities (23).

In 2003 Mussolino et al. from NHANES I noticed that the association of low hand BMD with allcause mortality in both black and white subjects was related to non-CVD mortality (9). In 2008, this finding was also described in NHANES III (including the addition of Mexican-Americans) where a strong relation between low total femoral BMD and non-CVD & non-cancer mortality was found (8). These findings were followed by a report by Johansson et al. (7) from MrOS Sweden: the authors described an association of total hip BMD with mortality in men which was multifactorial but mainly driven by causes other than CVD and cancer.

In the Rotterdam Study, it has been previously reported that a low femoral neck BMD (FN-BMD) was related to decreased overall survival in men (not in women), but the cause was not identified (3). Therefore, our first aim was to investigate the type of disease underlying the relation between a low BMD and decreased survival. Our second aim was to investigate if BMD was associated with calcification in the coronary arteries.

For both research questions we chose FN-BMD to facilitate the comparison with previous reports. FN-BMD can be classified into categories of normal BMD, osteopenia and osteoporosis (24), although this classification is also possible from BMD at other skeletal sites.

In Chapter 2 (25), we present the analysis of this relation in 7.834 participants from the first two cohorts of the Rotterdam Study (RS-I & RS-II) followed now for more than 10 years (17 years on average) and we reproduced the findings previously described by our colleagues with a follow-up of 5.4 years (3): each standard deviation (SD) decrease of FN-BMD (i.e., approx. 0.14 g/cm^2) is related to an increased all-cause mortality in men (but not women) by 9% [pooled HR men: 1.09 (1.04-1.15)]. Importantly, this result was not explained by fracture-related mortality.

With the purpose of improving the inference, the analyses were repeated excluding

subjects with fatal events within the first three years of follow-up. We found no evidence that early mortality attenuated our findings.

These results answer our first question:

Q1a. *Is femoral neck BMD still related to all-cause mortality in a longer follow-up study?*

Low BMD is related to increased all-cause mortality. In particular, we observed this relationship in men but not in women. The association described previously seems not to vanish with time, nor is it explained by early mortality. This argues against reverse causation – an argument commonly applied to justify the associations of bone with health outcomes other than fractures.

We also found that a low FN-BMD was especially related to chronic lung disease mortality in men from both cohorts and in women from RS-I. Each SD decrease of FN-BMD was related to an increased lung mortality of 80% in men from both cohorts [HR: 1.80 (1.40-2.31)] and of 72% in women from RS-I [HR: 1.72 (1.16-2.57)].

When we analyzed the results using T-scores of BMD (26) we found the following:

- Men with osteopenia at baseline had a hazard of dying from chronic lung disease during the 10-year follow-up of 121% compared with men with normal FN-BMD [HR: 2.21 (1.34- 3.62)];
- Men with osteoporosis at baseline had a hazard of dying from chronic lung disease during the 10-year follow-up of 1560% compared with men with normal FN-BMD [HR: 16.6 (5.84-47.0)];
- Women with osteoporosis at baseline had a hazard of dying from chronic lung disease during the 10-year follow-up of 490% compared with women with normal FN-BMD [HR: 5.91(1.20-2.90)].

Chronic obstructive pulmonary disease (COPD) (27) was the main cause among the lung mortality group. We found that each SD decrease in FN-BMD increased COPD mortality in 130% in men [HR: 2.30 (1.72-3.09)] and in 76% in women [HR: 1.76 (1.13-2.75)]. FN-BMD was found to influence COPD mortality independently of age, body mass index, smoking, inflammation, proxies of 25-hydroxyvitamin D status, vertebral fractures and prevalent COPD.

In our analyses, COPD mortality drove almost all of the association of low BMD with all-cause mortality in men from both cohorts, while we did not find associations with mortality due to cardiovascular diseases, dementia, infections, trauma or cancer.

A special emphasis in our analyses was given to assess the potential influence of vertebral fractures, as they are closely related to low FN-BMD (28). Vertebral fractures can induce pain and progressive kyphosis; both mechanisms are compatible with a restrictive pattern of lung function which is characterized by a decreased movement of the thorax with subsequent reduced lung volumes (such as vital capacity and total lung capacity) (29-33). In addition, these type of fractures can increase mortality in COPD patients (29,34). Nevertheless, we found no evidence for prevalent or incident vertebral fractures as an underlying mechanism for the relation between low BMD and COPD mortality. Nor did baseline COPD and glucocorticoid use influence our results.

- Bone mineral density and COPD mortality: an update in NHANES III

An important support for the causal inference of our results in Rotterdam Study was provided by AC Looker in 2014, who replicated in NHANES III an association between low FN-BMD and COPD mortality in non-Hispanic white men and women (35). The author also found low FN-BMD to be related to COPD prevalence. Progressive adjustments did attenuate somewhat the relation. Nevertheless, Looker concluded that there was enough evidence that confounding could not fully explain these results and, intriguingly, that the link between

low BMD and COPD appeared to be initiated many years before overt COPD development.

As previously mentioned, NHANES researchers had already in 2008 been able to identify that a low (total proximal) femoral BMD was related to all-cause mortality, not driven by CVD or cancer (8): subjects in the lowest BMD quartile displayed a 104% increased mortality due to a non-CVD & non-cancer cause [RR: 2.04 (1.24-3.35)].

After 16 years and in line with our data from Rotterdam, Looker identified this disease process to be COPD (32).

These findings answer the second part of our first set of questions:

Q1b. *Can we identify a specific disease through which survival is impaired by a condition of low BMD and explain this relationship, or is it all explained by fractures?*

COPD is the disease (identified in the Rotterdam Study and NHANES cohorts) for which survival is strongly influenced by a low BMD and that, in fact, drives most of the association between low BMD and all-cause mortality in men; this latter association is not explained by fracture-related mortality.

BMD and coronary artery calcification (CAC): rationale for this research in the absence of an association linking BMD and CVD mortality

In contrast to the absence of evidence of a relation between BMD and CVD mortality (21), metaanalyses assessing an association with arterial calcification (AC) had less heterogeneity, allowing the conclusion that BMD is inversely related to AC (36, 37). Most studies of this sort have been, however, cross-sectional and composed of post-menopausal women only.

As previously mentioned, AC is an independent CVD risk factor for fatal and non-fatal events (13, 20, 38, 39). Although the morphology and location of calcified lesions exert a crucial role in plaque stability (40), AC assessment provides useful clinical information for re-classification and risk management, especially for patients in intermediate risk categories (20).

Therefore, to answer our second set of research questions, we analyzed whether markers of bone health (FN-BMD; FN-BMD loss) (41) were related to calcification in the coronary epicardial arteries (CAC) in the Rotterdam Study. Previously, BMD loss in cortical compartments (metacarpal bone) has been related to aortic calcification progression in post-menopausal women from Framingham Study and from a Dutch population from Zoetermeer (42, 43); however, whether a similar relation could be identified between BMD loss and coronary calcification was uncertain.

In this context, it is important to note that excluding advanced CKD and severe primary hyperparathyroidism (44, 45), CAC occurs mainly in the intima and therefore reflects atherosclerotic burden.

In Chapter 3 (46), we describe the relation between BMD and BMD loss and CAC through cross-sectional, prospective and "hybrid" approaches. In the first approach, we tested whether FN-BMD was related to CAC; in the second approach, we assessed if CAC scores were related to fracture risk; and in the third approach, we analyzed whether FN-BMD loss (assessed in two points in time) was related to CAC (assessed in one point in time).

We found no cross-sectional association between FN-BMD and CAC. After applying competing risks models, CAC scores were not a risk factor for fractures. But in line with previous data (42,43), in 1.276 participants we found the following: a) each 1% annual FN-BMD loss was related to increased CAC scores in women (but not men) assessed both continuously [β: 0.22 (0.06-0.38)] and categorically [prevalence ratio (PR): 4% (1% - 7%)]; and b) the relation between FN-BMD loss

and CAC was constrained to women in the lowest 17*β*-estradiol levels (<16.4 pmol/L) and in the highest alkaline phosphatase levels (>76 U/L), suggesting a condition of marked hypoestrogenism and increased bone turnover as underlying factors for the association.

The consistent evidence of (mainly cortical) BMD loss and arterial calcification progression in women from our study and from previous research (42,43) requires, however, a careful interpretation. Through an elegant experiment, a Japanese group showed (47) that the bone-related proteins osteoprotegerin (OPG) and the Receptor Activator of Nuclear Factor κβ (RANK) are also expressed in human aortic endothelial and smooth muscle cells, reflecting the potential vascular responsiveness to RANK ligand (RANKL). RANKL increases the synthesis of bone morphogenetic protein 2 (BMP-2) in endothelial cells, stimulating the expression of osteogenic genes, such as *ALPL* (alkaline phosphatase), in smooth muscle cells and therefore favoring transdifferentiation into osteoblast-like cells (48). Therefore, RANKL increases calcification in vascular cells. Importantly, estradiol attenuates RANKL-induced calcification partly through an increase in Matrix Gla Protein, a calcification inhibitor that blocks BMP-2.

Consequently, the clinical context of marked hypo-estrogenism increases RANK/RANKL activation that induces a) osteoclast activation and mineral resorption in bone tissue (49) and b) BMP-2 activation and subsequent calcification induction in endothelial and smooth muscle cells (47).

Remarkably, the authors showed evidence that it is not serum (i.e., bone-derived) RANKL but instead locally expressed RANKL which is the underlying source for the described events (47).

A potential explanation for our findings that hypo-estrogenism appeared to underlie both processes of bone loss and AC in women but not in men could be the absent role for 17*β*-estradiol in AC process in men – as shown in a series of sequential experiments by Fitzpatrick et al. (50,51). On the other hand, serum

estradiol levels are much lower in postmenopausal women than men of the same age.

Moreover, there is clinical evidence supporting the crucial role of 17*β*-estradiol as an inhibitor of arterial calcification in women: a previous randomized controlled trial has provided evidence that hormone replacement therapy (HRT) decreases coronary calcification (52). However, estrogen exerts complex effects at the vascular level. Importantly, it must be added that HRT cannot be considered a therapy to decrease overall CVD risk, as the landmark HERS & WHI studies and Cochrane meta-analyses have shown the opposite to be true (53-55).

We conclude that our findings support an absent primary role for bone in the association between cortical bone loss and coronary calcification in women; instead, the available data suggest the mediation of low 17*β*-estradiol as the trigger agent for both pathological processes.

Therefore, the answer to our second set of question is as follows:

***Q2a**. Is femoral neck BMD related to coronary artery calcification? **Q2b**. In addition, is femoral neck bone loss related to coronary artery calcification? **Q2c**. Is there evidence that the presence of coronary calcification increases fracture risk?*

We found no evidence for an association between FN-BMD and CAC. However, in longitudinal analyses, there was a relation between FN-BMD loss and CAC which was evident only in women with the lowest levels of 17β-estradiol, suggesting its role as a mediator. In addition, our results do not support an association between coronary calcification and fracture risk.

P, BMD and fracture risk in the Rotterdam Study and MrOS: rationale for assessing bone outcomes

Albright and colleagues were among the first researchers to describe the phenotype of a child affected with walking difficulty, bone deformities, and multiple non-healing fractures, despite high doses of vitamin D, as the underlying rickets were induced by X-linked hypophosphatemia (56). The literature has been productive (57-59) since then in the description of similar cases and their clinical, biochemical and genetic profiles not only in hypophosphatemic disorders but also in their hyperphosphatemic counterpart (60, 61).

However, aside from Mendelian disorders, the association of P with bone outcomes has been remarkably absent in the literature, perhaps reflecting a tacit assumption that P levels not lying in the extremes of its distribution are not related to bone disorders.

Recently, the diagnosis of mineral and bone disorder in chronic kidney disease (CKD-MBD, previously renal osteodystrophy) has emerged as an exception to this gap in knowledge (62). CKD-MBD is composed of a dual condition of high P load (overt hyperphosphatemia in advanced stage) (63) and a severely increased fracture risk in comparison with subjects with normal kidney function. Depending on the CKD stage, fracture risk can increase fourfold, sixfold and even tenfold (64). Several authors have provided evidence for an important role of P in increasing fracture risk in CKD patients (65, 66).

In contrast to studying the role of P in CKD, whether P is related to BMD and fracture risk in the general population has been largely unexplored. This is despite a) the discovery of FGF23 in the year 2000 (67), b) the identification of osteocytes as the main site for FGF23 synthesis in the year 2003 (68, 69), and c) the overlooked concept that osteocytes compose 95% of bone cells (69).

More recently, FGF23 and α-klotho proteins have been considered the most

important axis to control serum P (or P load), even more so than PTH (70, 71).

In Chapter 4 (72), we present the results from a joint analysis from the Rotterdam Study I and II and the MrOS USA cohorts, where we found the following results:

a) P was not related to FN-BMD; it was negatively related to LS-BMD in men from the Rotterdam Study and in combined analysis.

b) P was positively associated with all-fracture risk with a stronger estimate in men but no evidence of sex-interaction. Each 1 mg/dL increase in P increased fracture risk in 52% in men [(HR: 1.52 (1.34-1.74)] and in 32% in women [HR: 1.32 (1.04-1.67)].

c) Higher P levels in men increased predominantly vertebral and wrist fracture risk and to a lesser extent hip and rib fracture risk; in women P increased humerus fracture risk as the only skeletal fracture site (**Table 1**).

d) In men only, normal serum P was consistently related to increased fracture risk.

e) Our joint data suggest a P threshold of 3.3 mg/dL (1.1 mmol/L) in men and 3.7 mg/dL (1.2 mmol/L) in women above which fracture risk starts to increase.

f) In men, P was associated with increased fracture risk across the entire range of kidney function and consistently also in CKD. In women, this relation was significant only in the group with normal kidney function (**Table 2**).

Table 1. Phosphate and fracture risk according to skeletal site and sex: pooled results from the Rotterdam Study and MrOS cohorts [source (72)]

	Men			Women		
Fracture site	**fx/total n**	**HR (95% CI)**	***p***	**fx/total n**	**HR (95% CI)**	***p***
Wrist	104/7439	**1.90** (1.20-2.99)	0.006	105/2497	1.18 (0.74-1.89)	0.477
Vertebral	219/7447	**1.73** (1.27-2.37)	0.001	116/2543	1.16 (0.73-1.83)	0.533
Rib	246/7441	**1.40** (1.05-1.88)	0.022	21/2556	1.09 (0.37-3.23)	0.873
Hip	265/7443	**1.36** (1.02-1.82)	0.037	77/2548	1.12 (0.65-1.93)	0.687
Humerus	83/6568	1.61 (0.99-2.62)	0.055	42/2539	**2.16** (1.06-4.40)	0.035

Table 1 highlights the differences for P in fracture risk according to sex and fracture site. In men, P increased fracture risk predominantly in trabecular-enriched bones, such as vertebra and wrist, where a rapid gradient of increasing trabecular bone content along the radius axis (e.g from <5% to >80%) has been shown (73, 74).

Apart from differences in power for the analysis across sexes, women displayed increased fractures with increasing P but this association was of weaker magnitude than in men and it reached statistical significance only at the humeral bone (75-77).

Table 2. Phosphate and fracture risk according to eGFR and sex: pooled results from the Rotterdam Study and MrOS cohorts [source (72)]

	Men			Women		
eGFR	**fx/total n**	**HR (95% CI)**	***p***	**fx/total n**	**HR (95% CI)**	***p***
>58 mL/min	1046/6435	**1.46** (1.26-1.69)	<0.001	349/2107	**1.37** (1.06-1.77)	0.016
<58 mL/min	167/946	**1.93** (1.42-2.62)	<0.001	46/235	1.10 (0.55-2.18)	0.790

Table 2 highlights the differences for P in fracture risk according to sex and CKD status. This relation in men was not only consistent also in CKD, but even stronger (I^2 across eGFR strata~60%) (78). In women, P was related to increased fracture risk only in those without CKD, although the small number of fractures prevents the drawing of definite conclusions.

From a physiologic perspective, a clear distinction must be made between the

association of P and fracture risk in participants without CKD (general setting) and P and fracture risk in patients who fulfill the diagnosis of CKD (CKD setting).

In the general setting, the findings in men of P being related to increased fracture risk at predominantly trabecular-enriched bones, and their consistency after constraining the analyses to normal P, allow the formulation of "osteocyte deficiency" as a potential underlying mechanism. This concept was coined by JESUS Delgado-Calle et al. (79), who showed that patients with hip fracture had a marked decrease (~95%) in *FGF23* expression in trabecular osteocytes in relation to decreased bone mass and/or increased osteocyte apoptosis.

Similarly, a condition of decreased "osteocyte density" (80) has been postulated where impaired fatigue microdamage detection and reduced canalicular flow can increase bone fragility independently of BMD.

In the CKD setting, different mechanisms can be postulated. The distinct eGFR threshold applied for CKD classification in our manuscript (58 mL/min instead of 60 mL/min) stems from evidence that at an eGFR of 58 mL/min several compensatory mechanisms in CKD progression are triggered, especially FGF23 release (81). As a result, hyperphosphatemia is a late phenomenon in CKD; however, there are adverse consequences of this "secondary hyperphosphatonism", including impaired bone mineralization and decreased 1,25 dihydroxyvitamin D synthesis (82,83).

Therefore, for the same P level across eGFR categories, the clinical context is different as a "normal P" within a CKD diagnosis implies that several complex compensatory networks with adverse consequences in bone have already been recruited.

- P and fractures: a note of caution on the interpretation of results after adjustment for FGF23 serum levels

The almost null modification after adjusting our findings for FGF23 levels do not imply an absent role of FGF23 in the association between P and fracture occurrence. It has been shown that there is an important discordance between *FGF23* expression and serum levels: even a decrease in *FGF23* expression in bone cells by almost half does not translate into changes of serum FGF23 levels (79, 84). This discordance has to be taken into account wherever serum FGF23 levels adjustments are applied to avoid oversimplification and incorrect interpretations.

According to our data, we can answer the third question as follows:

***Q3a**. Is serum P related to BMD in the general population? Are there differences according to skeletal site? **Q3b**. Is P related to fracture risk –even within normal ranges? If so, can a particular pattern in fracture-site be identified? **Q3c**. Are there sex differences for any of these outcomes?*

P was negatively related to LS-BMD in men. P was related to increased fracture risk, with a potential sex dimorphism in the fracture site, since there appeared to be a predominant effect in trabecular-enriched sites in men based on our data from Rotterdam and MrOS. Increasing P within the normal range was significantly related to fracture risk in men only, in whom a consistent relation was also shown in CKD. Replication of our findings regarding sex-differences in these relations is needed as well as more research into underlying mechanisms.

Phosphate and mortality: beyond CVD mortality

In Chapter 5 (85), we analyzed prospectively the relation of P with all-cause and cause-specific mortality in the Rotterdam Study I and II cohorts spanning more than 10 years of follow-up. In line with previous data (86, 87), we found that: a) P was associated with increased all-cause mortality in men [46% per 1 mg/dL

increased P (HR: 1.46 (1.29-1.69))] and b) despite having 15% higher P levels than men, P did not influence mortality in women.

The assessment of cause-specificity replicated previous evidence (87) of P as a risk factor for increased CVD mortality in men [66% per 1 mg/dL increased P (HR: 1.66 (1.29-2.14))]. Intriguingly, P was also found to be related to increased COPD mortality in men [344% per 1 mg/dL increased P (HR: 4.44 (2.08-9.49))].

The findings of P as a factor increasing all-cause and CVD mortality have been published previously (86, 88) and confirmed in meta-analysis (87). Furthermore, the sex difference has also been highlighted previously, especially by Onufrak and his group (86, 89).

Yet, we were able to identify for the first time in humans an association between P and lung disease mortality, which proved to be completely driven by COPD and, despite a smaller sample size than other causes of mortality within our data sets, showed a larger magnitude than CVD mortality ($p_{\text{heterogeneity}}$=0.016). This finding was not attenuated by multiple adjustments and was consistently observed after constraining the analyses to participants with normal serum P levels.

As current smoking was related to increased P levels, in addition to smoking adjustments, we stratified analyses and found that each 1 mg/dL increase in P was related to an increase of 459% in COPD mortality in former smokers [HR: 5.59 (2.21-14.1)]. Although the analysis was constrained to a smaller sample size, we found no significant relation in current smokers [HR: 2.64 (0.38-18.3)].

- Potential underlying mechanisms

For COPD mortality:

Although the relation between P and COPD mortality is novel in human epidemiology, a similar observation has been described in basic research since the discovery of the *klotho* gene. In fact, it has been systematically documented

that lung emphysema is a key component of the premature ageing phenotype of rodents with genetically-induced severe hyperphosphatemia (90-94). Normal *klotho* expression is required for postnatal alveolar integrity (93). Both *fgf23*$^{-/-}$ and *klotho*$^{-/-}$ mice (phenocopies) develop postnatal emphysema that remarkably, can be rescued with restoration of normophosphatemia, either through genetic (*NaPi2a*$^{-/-}$) or dietary interventions (90). Importantly, sex-differences in rodent models have been described in the phenotype rescue: while normophosphatemia rescues the aging phenotype in male mice, it has no effect on female mice (91).

In addition, animal models and cell culture experiments have shown that a high P medium [3-5 mM] directly induces oxidative stress and apoptosis in human lung epithelial cells; of note αKlotho protects against this damage (95, 96). In physi logical conditions, high phosphorus intake decreases expression of *klotho* (91), which is required for alveolar integrity as its absence induces lung emphysema (93). *KLOTHO* expression has been recently described in humans tissue (97), especifically in lung tissue (98) and found to be decreased (99) in COPD patients versus smokers (p<0.05) and versus non-smokers (p<0.001).

For CVD mortality:

There is evidence from basic (48), epidemiologic (18) and clinical research (100) that high P can induce arterial calcification (AC), especially in the media. Such an association has been extensively shown in CKD (101) but scarcely investigated in the general population (102). High phosphorus intake acutely leads to calciprotein particles formation (103), one of the first steps in AC. In addition, compensatory FGF23 rise in CKD induces myocardial hypertrophy (104). Nevertheless, whether an increased but within normal range P can induce AC has been unclear, but recent research from our group has found evidence for normal P to be causally related to AC in the general population (**Chapter 6**; unpublished data).

With these cumulative results, we can answer the following lines to the fourth question:

***Q4a**. Is there evidence that higher P levels - but within normal range – are associated with all-cause mortality in data from Rotterdam? If so, what specific diseases are involved, or is it driven by cardiovascular mortality? If not, is there evidence of P influencing other causes of mortality, as suggested from findings in animal studies? **Q4b**. Are there sex differences in our results?*

We found evidence that P, even at normal levels, was related to decreased survival in men. As previously shown, P was associated with increased CVD mortality, but, in addition, our data showed a consistent relation of P with COPD mortality - not seemingly explained by confounders. This observation is novel for human studies but not for animal models with severe hyperphosphatemia. Once more, our results displayed marked sex differences, with no associations seen in women despite higher P.

Increasing but normal P and coronary artery calcification (CAC): evidence for potential causality

Patients with severe hyperphosphatemia develop a premature ageing phenotype (105) with AC as one of the key components of the clinical cluster, leading to increased risk for CVD events and death (20). Whether normal P is causally related to AC has not been clarified. If confirmed, it would become not only a pathologic mechanism underlying the relation between higher P within the normal range and CVD mortality; however, it would also mean that an increasing P even within normal range should be considered a CVD risk factor, as postulated 10 years ago (106).

Chapter 6 is composed of two sections. In the first, we describe a phenotypic association between P and CAC, an AC process that occurs mainly in the intima and is specific of atherosclerosis (20) and predictive of coronary heart disease

(107). Hyperphosphatemia has been related to CAC, but its association with normal P has yet to be fully elucidated (102). We hereby show this association in subjects even without hyperphosphatemia, CKD and prevalent CVD.

In the second section, we apply Mendelian Randomization (MR) (108), an approach to improve causal inference. Our results indeed supported a causal relation between P and CAC in the Rotterdam Study. Causality was also supported for subjects without hyperphosphatemia, CKD, and prevalent CVD. As sensitivity analyses (109), we found that one SNP lying close to the *ALPL* locus (110, 111) exerts a major influence on our results – supporting an important role for alkaline phosphatase (ALP) and P release from pyrophosphate (PPi) on CAC (112).

Two main pathways of P-induced calcification have been described in the coronary bed: 1) a passive deposition of calcium and P, regulated by ALP/PPi/P axis; and 2) an active process of osteoblastic differentiation of vascular pericytes and calcifying cells that are able to synthesize matrix vesicles (MV), which start the mineralization process. Current evidence has shown the presence of ALP/PPi/P in MV surfaces of atherosclerotic plaques (40), closely linking both mechanisms of calcification in CAC and providing a biological explanation for our results.

Remarkably, MR sex-stratified analyses showed a more consistent association in men. Previous literature (85-87, 89) and a study from this thesis [**Chapter 5**] have systematically reported stronger (or unique) results of P in men for all-cause and CVD mortality and atherosclerosis. These results could be seen as counterintuitive because postmenopausal women have higher P and lack a protective effect of high 17β-estradiol levels on AC as shown in premenopausal women.

With these results, we can answer the fifth question in the following terms:

***Q5a**. Are P serum levels causally related to coronary calcification? If so, are the results driven by hyperphosphatemia, CKD or prevalent CVD? **Q5b**. Can we find evidence at the MR level of a sex-difference, strongly suggested by*

epidemiological papers on the association between P & coronary calcification, CVD morbidity and CVD mortality?

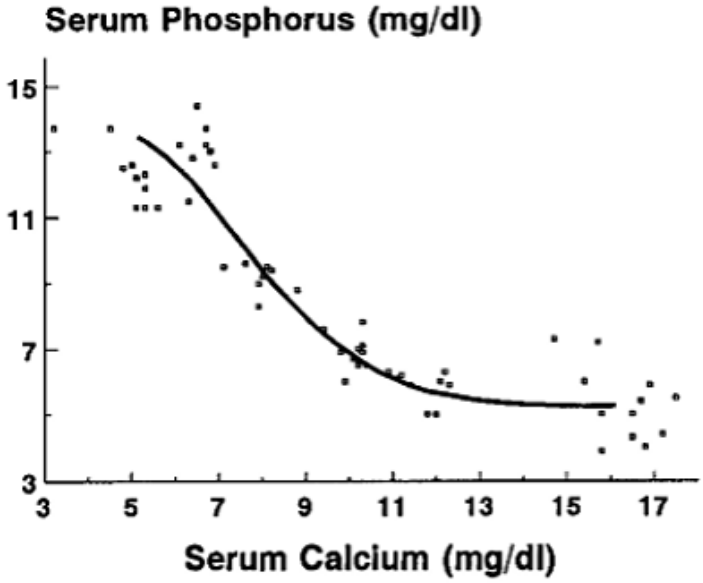
Our results from MR analysis support a causal role of serum P levels in coronary artery calcification in the general population, meaning that our findings were not driven by hyperphosphatemia, CKD or prevalent CVD. The sex-stratified MR analysis strongly suggests a more consistent causal association in men than in women, which is in line with the systematic sex-difference in the association between P and CVD outcomes described in the literature.

- Sex-difference in the association between P and CVD outcomes

We can only speculate whether the association between ionized calcium and P plays a role in the sex differences for P and CVD outcomes as we [Table 1, **Chapter 6**] and others (113-116) have found an inverse relation between them in women but not in men; moreover, an inverse reciprocal relation seems necessary to keep a constant calcium*phosphate product (Ca*P product) in serum (117). Animal studies carried out in rodent models have shown an inverse relation between Ca and P across a wide range of values [**Figure 1**] (117). Human studies have also shown an inverse relation between ionized Ca and P and, importantly, the available data support a sex dimorphism in this association, as several authors (113-116) have found that ionized calcium decreases with increasing P in women, but not in men (116).

It must be added that recent research has reaffirmed the importance of the Ca*P product, as it tightly correlates with calciprotein particle formation - a key step in AC pathogenesis (103, 118). Several authors, such as Parfitt et al. & Felsenfeld et al., have shown that hyperphosphatemic states induce skeletal resistance to PTH, with decreased calcium release from bone (117). The status of decreased calcium release in the presence of hyperphosphatemia has been proposed as an important mechanism to achieve the constancy of Ca*P product. Whether there is sex dimorphism in this mechanism warrants further research.

Figure 1. Reciprocal relationship between serum calcium and phosphorus in animal studies.



The figure shows the inverse relation between serum calcium and phosphorus after PTH infusion found in animal studies on rat models. (Reproduced with permission from authors and with authorization from the *Journal of the American Society of Nephrology*. Source (117)).

- Phosphate as the factor underlying the calcification paradox: the importance of the bone compartment

More recent studies applying quantitative computed tomography (QCT) have compared the independent contributions of trabecular versus cortical bone compartments in relation to arterial calcification (AC) and have shown what seems to be an exclusive association of trabecular bone compartment (volume, trabeculae number and separation), especially in older men (14, 119-121). Indeed, several authors have described elderly men as the only subgroup displaying a consistent inverse relation between trabecular bone and AC after adjustments (119, 120) and suggesting a role for trabecular bone deterioration in arterial calcification.

No association between QCT-assessed cortical bone compartments and arterial calcification has been identified in either sex (119, 120).

Findings from **Chapters 4** and **Chapter 6** from this thesis support the concept of a role of trabecular bone in arterial calcification pathogenesis. First, the demonstration of a significant inverse association between LS-BMD and P in men only - without such an association with FN-BMD (72) suggests a predominant role of trabecular osteocytes (over cortical osteocytes) as a source of FGF23 and subsequent regulators of P levels in men [**Chapter 4**].

Second, the demonstration of a potential causal role of P in coronary calcification in men and women (more consistent in men) through Mendelian Randomization and in the absence of CKD and hyperphosphatemia [**Chapter 6**].

There is supportive evidence from basic and clinical research that strengthens this postulate, especially a preferential location of FGF23-secreting osteocytes in trabecular bone, consistently described in studies including *FGF23* expression data and performed in animal models (122, 123), in both diseased subjects (79, 124-126) and in healthy controls (125).

This preliminary evidence leads to a postulation of P as the main link between (trabecular) bone and AC. Importantly, experts in the research field, such as Demer & Tintut (127), have suggested three pathological mechanisms as pathways to explain the association between arterial calcification and osteoporosis –previously known as the "calcification paradox" and termed by the authors as the "bone-vascular axis": 1) arterial calcification promoting bone loss; 2) bone loss promoting arterial calcification; and 3) a common underlying mechanism, such as low estradiol (previously commented on but not further elaborated here as it discards a primary role for both bone and blood vessels).

The second pathological mechanism has more validity than the other two as bone resorption can release several factors active in the vascular system. The authors

postulate that P could act as one such mediating agent. Of course, other bone-derived substances could also exert a role, but current evidence supports P released from bone as a factor able to exert arterial calcification, based on experimental (48), epidemiological [(102) and **Chapter 6**] and clinical (100) data. As additional evidence for P as a good candidate to underlie the bone-vascular axis, other potential bone-derived candidates exert rather anti-calcifying properties in the vascular system (e.g., FGF23, osteopontin) (128-130).

In summary, the main results from Part I of this thesis provide evidence for an association between low BMD and P in COPD mortality. In addition, P is related to fracture risk and CVD mortality and causally linked to coronary calcification, even at normal levels. Most results were characterized by a marked sex difference, with more prominent associations seen in men and attenuated or null results in women. We aim to explore further this finding in the next section of this thesis.

Part II. Sex differences in calcium and phosphate levels

Clinical research from decades ago has systematically reported a sex dimorphism in the epidemiology of CVD (131). In addition, differences in serum levels of minerals across men and women, especially for calcium (Ca) and P, have been previously reported (132, 133). For example, Haglin et al. (132) found a decreased P level in men of 8% in comparison to women (50 years of age in average).

More recently, the literature has described a relation of these minerals with hard outcomes – including mortality – characterized by a marked sex difference driven by stronger or unique findings in men. Cohorts and post-hoc analyses of the RCTs such as NHANES, Framingham, the Tromsø Study and CARE have reported associations between Ca & P with CVD events in men only. This sex-difference has been found to be more consistent for P than for Ca (87, 106, 134).

In a similar way, several studies in this thesis reported important sex-differences in the relations between BMD & P with several outcomes and traits. Concerning

outcomes, the following can be mentioned: BMD & P in all-cause mortality [**Chapters 2** and **5**], P in CVD mortality [**Chapter 5**], BMD & P in COPD mortality [**Chapter 5**], P in fracture risk [**Chapter 4**] and P in coronary artery calcification also in the Mendelian Randomization analysis [**Chapter 6**]. Concerning traits, the association between P and LS-BMD in the Rotterdam Study showed a consistent sex difference [**Chapter 4**].

Total calcium & phosphate levels in the Rotterdam cohorts: potential role for 25OHD and gonadal steroids in the sex differences

In Chapter 7, we investigated differences in Ca and P levels between sexes in the Rotterdam Study. The results from 9.253 participants showed that Ca levels were 1% higher and P levels were 6% higher in women when compared to men (Ca: 9.76 mg/dL vs 9.64 mg/dL; P: 3.16 mg/dL vs 3.63 mg/dL) and, as a consequence, the Ca*P product is also higher in women than men. These results correspond well with previous observations (132).

Several studies have shown that Ca and P levels and albumin differences between sexes oscillate according to age and menopausal status (135). Therefore, we investigated whether sex differences in Ca and P were already present at younger ages in the patients assessed routinely at Erasmus MC spanning a wider range of ages than the Rotterdam Study (older than 45 years only).

In Chapter 8, we analyzed laboratory data from the Hospital Information System of Erasmus MC, Rotterdam, on Ca, P and albumin levels from patients of 1-17 years, 18-44 years and > 45 years. Three such age-stratified "cohorts" were selected, differing by year of sampling (2005, 2010, and 2014) and including a total of 15.215 patients. We observed that serum levels were consistently higher for both Ca (2%) and P (7%) in women than men in the age category above 45 years, while no differences could be observed at younger ages. The higher Ca and P levels in women than in men of this age group in the large Erasmus MC patient series were also observed in the Rotterdam Study in a similar magnitude for Ca

levels, while P levels were 15% higher in women than in men.

We went on to test for a potential role of serum levels of 25-hydroxyvitamin D (25OHD) and gonadal steroids in this dimorphism. These differences were not explained by 25 OHD nor by $1,25(OH)_2D_3$, alkaline phosphatase, and albumin levels. Nor did we see consistent effects of adjustments for gonadal steroids in Ca levels when compared across subgroups diverging in fasting status prior to blood drawing.

Finally, we noticed that the adjustment for testosterone levels attenuated the sex difference by 38%. In contrast to expectations (136, 137), the adjustment for 17*β*-estradiol levels has no influence in the sex difference. Previously, the MrOS US study found a role for testosterone as a partial explanation of the sex differences in P levels (136). This influence of testosterone could reflect its relation to aromatization into 17*β*-estradiol in adipose tissue (138) and the phosphaturic actions of estradiol through direct downregulation of renal NaPi-IIa transporters (139), and through enhanced FGF23 synthesis (137).

We therefore answer the sixth question as follows:

***Q6a**. Do the differences in calcium and P span several age categories? Are they explained by gonadal steroids? **Q6b**. Can we find evidence in our data of another potential mechanism underlying the sex differences in calcium and P levels?*

Our data provide evidence for a sex difference in Ca and P levels above 45 years. Our results confirm the 1-2% higher levels of Ca and the 7-15% higher levels of P in women compared to men, and showed that this difference is only apparent above 45 years of age and not at younger ages. 17β-estradiol serum levels do not seem to influence this difference, while testosterone partially explains the higher P levels in women. Our results do not highlight additional mechanisms to explain the sex difference in serum calcium and P levels.

Part III. Genetic studies in relation to bone and phosphate

- **Exploring unusual genetic causes for osteoporosis and fractures: a Mendelian approach**

The integration of the inheritance pattern proposed by Fr. Gregor Mendel from his experiments with peas (140) with the observation of continuous variation induced the postulation of the infinitesimal model, published in 1918 (141) and later referred to as "the polygenic model". Most complex traits were initially proposed to follow such model as underlying genetic architecture. This meant that a substantial fraction of their variance is explained by the joint effect of a large number of single nucleotide polymorphisms (SNPs) exerting each one a minuscule effect. More recently, it has become evident that most traits are causally determined by a few thousand loci (142) – yet with large heterogeneity, according to trait category (143).

BMD is no exception: the largest genome-wide association study (GWAS) to date identified ~600 independent SNPs that account for 20% of its population variance (144). However, clinical data have shown that not all BMD variation is attributable to SNPs, as rare mutations in several genes can exert a large influence on BMD, usually detrimental and seen in families with pedigrees of segregating disease. Examples of these "major genes" are *COL1A1* [type 1 collagen synthesis], *WNT16* & *LRP5* [WNT signaling pathway] and *TNFRSF11, TNFRSF11A & TNSF11B* [RANK/RANKL/OPG pathway] (145).

We hereby present a cluster of exceptional cases of severe osteoporosis identified from general practice; their clinical evolution highlights the fact that, although not frequent, Mendelian disorders of BMD can exert a large impact on bone health.

<u>PLS3: the first identified cause for X-linked osteoporosis</u>

Chapter 9 (146) is composed of the presentation of 5 families with early-

onset osteoporosis and high fracture incidence in men but milder or no clinical presentation in women – highly suggestive of an X-linked pattern of inheritance. After testing for common mutations of osteogenesis imperfecta proved negative (*COL1A1* and *COL1A2*), a large collaborative effort among several bone groups identified the gene, mapped it to the X-chromosome, and demonstrated causality.

Though the setting of X-linked osteoporosis has been cited previously, to the best of our knowledge, this is the first study that confirms this inheritance pattern in osteoporosis.

The clinical picture can be summarized as follows. Most men from the 5 (outbred) families were affected by fractures since early childhood, and the pattern of severe bone fragility continued throughout life to the point of gathering multiple fractures (even up to 20) by early adulthood. No clear clinical signs of osteogenesis imperfecta were noticed, with the exception of joint laxity and short stature in isolated cases. Low BMD was present since early age, with Z-scores substantially below -2.0 SD; bisphosphonates proved to be a useful therapy in most of them in terms of BMD.

In women, the clinical picture was heterogeneous, ranging from no bone-related pathology to several fractures, but at later presentation and in fewer numbers than men. BMD values ranged in most cases from normal to the osteopenic range; however, osteoporosis was uncommon. Similar to men, no clear signs of osteogenesis imperfecta were noticed with the exception of occasional joint laxity

- Summary of the *PLS3* genetic findings

X-linked whole exome sequencing revealed a frameshift mutation in exon 3 of *PLS3* [which encodes for plastin] in three male patients from family 1. *PLS3* maps to Xq23. Sanger sequencing of *PLS3* exons confirmed this finding, identified the same mutation in three additional affected men, and identified 4 novel pathogenic variants in 95 men from families 2 to 5, including non-sense, frameshift, and

splice-site mutations.

In addition, a previously described *PLS3* variant (rs140121121; MAF 1.3%) was identified in five affected men. This variant was *de novo* genotyped in the RS cohorts to study the influence of more common genetic variation in the *PLS3* gene on bone phenotypes in the normal population, in contrast to the few families with very severe bone phenotype due to the rare mutations. It was shown that heterozygous women who were carriers of the risk allele (A) displayed a higher odds ratio for fracture than non-carriers [OR 1.95 (1.39-2.74)]. A lower LS-BMD was also observed in such heterozygous women. Perhaps constrained in power due to a small sample size, no consistent bone effects were shown in hemizygous men.

Linkage analysis was carried out to test for cosegregation of the discovered mutations in *PLS3* and the phenotypes at a genome-wide scale. Only families 1 and 2 contributed to the calculation of the lod score, which was estimated to be 3.40. Such linkage findings are consistent with a high probability ($p<4.9x10^{-5}$) that the identified variants are causative factors for the bone phenotype of low BMD and increased fracture risk (147, 148).

- Confirmatory findings at the functional level

Through *pls3* knockdown in zebrafish, it could be demonstrated that plastin plays a key role in skeletal development, as affected larvae showed severe craniofacial dysplasia and malformations in body axis and tail. All skeletal defects could be rescued by dose-dependent injections of human *PLS3* mRNA, proving causality of this gene in the bone phenotype.

More recent work, including bone histomorphometry on patients with PLS3 mutations, has revealed a low bone turnover pattern and prominent focal areas of osteoid accumulation, suggesting a role of *PLS3* in local bone mineralization (126). In addition, a substantial decrease in *FGF23* expression has been noticed,

and, as *FGF23* expression was found only in trabecular areas in patients with *PLS3* mutations, cannot be explained by cortical osteocyte apoptosis, which has been shown to be increased in patients harboring *PLS3* mutations (126). The decrease in *FGF23* expression can not be explained by increases in *DMP1* either, which is a regulator of the mineralization process that has been shown to decrease FGF23 production by bone cells through FGF receptor signaling (149).

LRP5 mutations and osteoporosis diagnosed at pregnancy

In Chapter 10 (150), we describe the case of a young woman with very low BMD who was suspected to have pregnancy and lactation associated osteoporosis (PLO) before genetic analysis was performed.

The clinical picture was characterized by severe back pain during her late pregnancy and she was subsequently found to have very low BMD on a DXA scan [Z score L_2L_4: -5.6 SD; FN: -3.9 SD] and three vertebral fractures. She had unspecified congenital unilateral blindness and a mild form of bilateral exudative vitreoretinopathy. No secondary causes of low BMD could be identified. Her mother had low BMD and her brother had early-onset fractures. Her father was deceased.

- Pregnancy and lactation osteoporosis

Pregnancy and lactation are associated with a decrease in (mainly trabecular) bone content estimated in 5-7% (151); both osteoclast-mediated resorption and osteocytic osteolysis have been described as pathogenic mechanisms (152). BMD loss during lactation can reach 1-3% per month, but longitudinal studies have shown (almost) full recovery in BMD. Consistently, parity and lactation are not risk factors for incidental fractures at the population-based level (153). Although long bones do not recover fully, there is a compensatory increase in their volume and in their cross-sectional diameters (154). As a consequence, the strength of long bones is not permanently decreased after each reproductive cycle (154).

In rare cases, there is a more severe decrease in BMD with microarchitectural changes, which cause an increased incidence of fractures, preferably at the spine.

Fractures are uncommon in normal pregnancy (151). However, when they do occur, a complete clinical examination should be undertaken, as a condition of low BMD prior to pregnancy is highly probable. Indeed, this case shows that a preexisting low BMD condition due to a genetic disease might be an important risk factor for the skeleton to decrease the tolerance to the physiologic bone content loss induced by pregnancy and, especially, by lactation.

- Summary of genetic findings

Firstly, *COL1A1* and *COL1A2* analyses were not compatible with a diagnosis of osteogenesis imperfecta. Secondly, considering the joint involvement of bone and ophthalmic tissues, DNA analysis of low density lipoprotein receptor-related protein 5 (*LRP5*) was carried out and showed compound heterozygosity for c.1519G>A (p.Gly507Ser) and c.3758G>T (p.Cys1253Phe) mutations, each predicted to affect conserved aminoacid residues. Her mother and brother were subsequently shown to be carriers of the *LRP5* c.3758G>T mutation.

In 1996, Gong and coauthors identified *LRP5* as the causative gene of osteoporosis-pseudoglioma syndrome (OPPG; OMIM 259770), an autosomal recessive disorder characterized by severely low BMD and congenital blindness (155). This dual affection is due to the key roles of LRP5 on both WNT and Norrin signaling; the latter is an atypical WNT ligand crucial in retina vascularization (156, 157). Obligate carriers can have low BMD, showing that bone effects of LRP5 can be dominant.

In addition to OPPG, *LRP5* mutations can induce familial exudative vitreoretinopathy (FEVR), a heterogeneous disorder with milder bone involvement than OPPG. Both mutations have been found to segregate in families with OPGG or with FEVR (158,159). To the best of our knowledge, functional analyses

have not been carried out; nevertheless, novel machine learning techniques have predicted the pathogenicity of p.Cys1253Phe (160), a mutation predictive to rupture disulfide bonds. Qin et al postulated that there is a continuous clinical spectrum spanning the diagnoses of OPPG and FEVR (161). Indeed, this seems to be the case for our patient and we found the spectrum of OPPG/FEVR underlying the diagnosis of PLO.

We can answer the seventh question as follows:

***Q7a**. What are the genetic variants underlying the phenotype of these patients? Are they lying in BMD annotated genes? If not, what additional evidence can be obtained to support causality? **Q7b**. What lessons useful for the common clinical practice can be learned from these cases?*

Next to clear polygenicity of BMD genetic architecture, several Mendelian disorders can exert a strong detrimental influence in bone strength. A pathogenic variants in PLS3 at the X chromosome has been found for the first time underlying an X-linked pattern of inheritance of severe osteoporosis with fractures; for the pregnant woman who presented with osteoporosis, heterozygous compound LRP5 variants were found to underlie a diagnosis of PLO.

Several lessons to highlight from these two genetic causes of osteoporosis are: a) men are often understudied and underdiagnosed for BMD-related pathology, but we hereby showed that a variant in an X-linked gene that had never been associated with osteoporosis before causes a severe bone phenotype in men; b) a solely low BMD diagnosed in pregnancy or lactation without fractures is not in itself mandatory of bone-sparing treatment, as in most cases a (almost) complete BMD recovery is achieved following the transient loss; and c) fracture occurrence is uncommon during pregnancy or lactation. Its occurrence, together with a not-recovering low BMD, should necessitate a complete clinical workup to exclude secondary factors and the consideration of an underlying genetic bone disease. Moreover, the risks of rapid bone loss during breastfeeding in patients with

osteoporosis and/or fractures should be discussed with the patient. In cases of a preexisting low BMD before pregnancy, breastfeeding is discouraged or advised to be limited to short duration.

Genome-wide Association Study of human serum phosphate levels: a large Biobanks approach

Lately, the availability of large-scale biobank resources has made it possible for an exponential increase in sample sizes to analyze phenotypes and genotypes that have been measured in such biobanks. This has also led to GWAS of ever increasing sample size, and, therefore, to increase the discovery of more causative genetic variants and to explain a larger fraction of the genetic variance of the available phenotypes. In particular, the UK Biobank (162) contains many phenotypes that are relevant to bone research. This section focuses on the genetic architecture of serum phosphate levels (P), whose measurements were available in close to 400.000 subjects.

In Chapter 11, we analyzed GWAS data in relation to serum P levels from 392.655 participants from the UK Biobank (162). We excluded those with a glomerular filtration rate below 39 mL/min, as below this threshold the prevalence of hyperphosphatemia is markedly increased (81). The implementation of a mixed model regression allowed us to keep related subjects (close to 33% of the total of the UK Biobank) and is robust against population stratification. In addition, we allowed for the inclusion of the non-infinitesimal model, since most traits seem to be explained by a few thousand causative loci and not by the totality of SNPs, as implemented in standard mixed models (142). We applied a significance threshold of $5x10^{-9}$, as previously suggested for large datasets with many phenotypes and genetic variants (163).

After applying approximate conditional analyses through GCTA (164) to identify independent variants, we were able to find 264 genetic variants for serum P levels, explaining 7.62% of its population variance. The genetic variants included 261

independent autosomal SNPs, three SNPs in the X-chromosome in men and one SNP in the X-chromosome in women (the latter corresponds to one of the locus in men). The top hit (rs9469580; 6:33715837) lies in the short arm of chromosome 6, in the region flanking the Major Histocompatibility Complex (MHC) (165). This common SNP has a minor allele frequency (MAF) of 0.08, a β of 0.195 and a p value of 1.6 x10^{-468}. There were 34 primary independent signals in chromosome 6, nine of them were flanking the large MHC region (24-36 Mb, hg19). This region has been identified as genome-wide significant in previous GWAS (110, 111) but has not been identified as the top hit until now.

When restricting the analyses to a subset composed of White-British ancestry subjects (n=354.798), determined by UK Biobank as self-reported White British ancestry and after applying a Bayesian outlying algorithm based on the first six PCs (166), we obtained similar results.

Estimates for P explained variance are ~ 7.62%, according to the formula described by Visscher and colleagues (167), and when applying the infinitesimal model (the standard approach in classic mixed models) the estimate for P explained variance was ~ 5%. There was an important increase in this parameter since the first GWAS, that described 1.5% of explained variance in P based on analysis of 16.264 subjects (110).

Our GWAS could replicate previous findings in the locus near *ALPL* and *FGF23*, genes known to exert important effects in P. But the vast majority of the novel hits were lying not close to annotated genes previously known to be involved in controlling P serum levels. Furthermore, our results from a Bayesian statistical fine-mapping approach in the three main associated loci confirmed the role of *ALPL* and *FGF23* in P levels but strongly suggested that other genes underlie P homeostasis. Remarkably, fine-mapping results within 6p21.31 (the region flanking the MHC that showed the strongest association of our GWAS) strongly suggested that both *IP6K3* and *ITPR3* play key roles in determining serum P levels. This therefore opens up the description of new biology underlying P homeostasis.

Our results provide a slight better fit for the non-infinitesimal model, suggesting that P genetic architecture in humans is probably defined by a few thousand loci across the genome, instead of the classic assumption underlying standard mixed models that all variants are causal, each one with a minuscule effect on the trait or disease (142, 168, 169). Although there is an important fraction of missing heritability to be explained, the identified variants can be used in Mendelian Randomization studies to test for causality in the associations between P and disease. Further research is warranted to explore the mechanisms determining serum P levels for most of the 264 hits we found, especially the inclusion of genomic annotations in the fine-mapping approach and the performance of trans-ethnic meta-analysis, highly useful to leverage LD patterns in the MHC region (170,171).

Therefore, we answer the last set of questions in the following lines:

***Q8a**. What are the common genetic determinants of P levels in humans? **Q8b**. Are we able to identify low frequency or rare genetic variants with large effects? **Q8c**. Is there evidence of a noninfinitesimal architecture for P? **Q8d**. Can we find evidence of a sex-dimorphism in the genetic structure for serum P levels?*

Through this large-scale GWAS, we identified 264 genetic variants explaining 7.5% of the genetic variance of serum P levels. We were able to replicate the ten previously known hits, and quadrupled the variance explained. We found enrichment for low frequency variants, but not for rare variants. As expected, our results were suggestive of a better fit for the non-infinitesimal model for P genetic architecture. Most identified variants have no evidence of sex-dimorphism.

Future Directions

The studies described in this thesis have shown a role for bone and serum P on important outcomes, such as coronary artery calcification and mortality, including COPD mortality. Two important strengths of our results are a) they stem from population-based cohorts and b) the findings that a decreased BMD and increased

P are associated with adverse consequences on health even at a common fluctuation range. In summary, our findings were:

- a low BMD lying within the category of osteopenia was already found to be associated with COPD mortality in men;
- an increasing but normal P was related to increased fracture risk, lower lumbar spine BMD, CVD mortality and COPD mortality: all of these findings were constrained to men. In women, serum P was also positively associated with all-fracture risk but with weaker estimate than in men and this relation was not significant when restricting analyses to normal P levels.
- an increasing P within the normal range was phenotypically related to coronary artery calcification in both sexes. However, our data supports causality for this association in men but not in women, according to MR analysis.

Simply put, there seems no need of extreme variations on bone and P to exert impact on public health – certainly not in men. Based on our findings, we hereby summarize what potential remedial measures could be applied from both a preventive and a therapeutic perspective.

❖ Low BMD, P and COPD mortality

Considering that 80% of P is stored in bone, that bone-derived FGF23 is critical for P homeostasis in health and disease (71) and that osteocytes compose 95% of bone cells (172), the findings of:
a) an association between low BMD and COPD mortality and
b) an association between an increasing (yet normal) P with COPD mortality

can be theoretically connected. A decrease in BMD [proxy of bone mass in fully mineralized bone (173)] implies a decrease in osteocyte number and density and, therefore, a decrease in FGF23 synthesis (79, 80) with the subsequent reduction in P renal excretion (174-177).

Two potential measures to mitigate consequences for COPD can be suggested, as follows:

- 1) Measures to prevent a decrease in FGF23 synthesis

First, COPD patients should be screened for a low BMD condition. Our results were suggestive of a dose-effect for BMD decrease and the hazard of dying of COPD was found to be particularly high in subjects with baseline osteoporosis. Low BMD in general is an undertreated and neglected condition (178); this statement is unfortunately also valid in COPD despite its highly prevalent osteoporosis associated condition (~40%) (179-181), especially when the prevalence of vertebral fractures is taking into account.

The proper assessment of vertebral fractures should be emphasized due to the restrictive pattern in ventilatory mechanics and increased mortality that they impose in COPD (29,31). In particular, the high prevalence of atraumatic vertebral fractures in stable COPD patients (~36%) (180) – especially in men with COPD (182), the high risk of incident vertebral fractures during COPD progression (183, 184) and the fact that the increase in osteoporosis prevalence during COPD progression is due mainly to vertebral fractures and not to BMD decrease (183), warrants further research to test for a potential role of trabecular bone quality deterioration (manifested clinically as vertebral fractures) in COPD progression.

Some previous meta-analyses and individual studies have found or suggested that antiosteoporosis medication may be associated with decreased all-cause mortality (185-188). Although Cummings et al (189) did not find a statistical significant advantage for anti-osteoporosis treatment on survival overall, the authors described a suggestive potential benefit of nitrogen-containing bisphosphonates. Consistently, a recent mediation analysis study has shown a benefit in survival for nitrogen-containing bisphosphonates and, in addition, this benefit was mostly explained by a reduction in bone loss and only partially explained by fracture prevention (190).

A recent study provided evidence of benefit of inhaled bisphosphonates in mice models with elastase-induced COPD: the inhaled nitrogen-bisphosphonates alendronate and risedronate decreased emphysema through a macrophage-mediating mechanism (191,192). In addition, alendronate also showed a benefit in smoking-induced emphysema. If these findings can be translated to human populations awaits further research. Considering some epidemiological evidence that nitrogen-containing bisphosphonates may decrease mortality and this new line of evidence from animal studies, it will be of interest to investigate whether this type of antiosteoporosis medication may offer a survival advantage in COPD patients.

- 2) Measures to decrease P burden

Although not a topic of this thesis, high phosphorus intake correlates to serum P levels: more precisely with time-average P levels (193). The European Food and Safety Agency (194) called for an evaluation of potential adverse effects of P added in food additives. High P intake may cause an acute decrease in *KLOTHO* expression with potential adverse cardiovascular and renal effects (91,103). The evaluation of the effects of high P diets is a challenge, as highly-absorbable phosphorus from food additives is not commonly stated in food labels.

There is suggestive evidence to link high phosphorus intake with adverse outcomes: a) NHANES data showed that phosphorus intake increases all-cause mortality not explained by CVD (195); b) a large-scale diet study proved high cured meat intake (expected to have high phosphorus) to increase COPD risk in men (196); c) high cured meat intake is also associated with worsening of spirometrically-determined lung function and in COPD hospital readmissions (197). Whether these are effects of high phosphorus content in meat remains to be determined.

High phosphorus intake can in theory impair kidney function (even in healthy subjects) through tubular damage and nephron number decrease (103), establishing therefore a potential vicious cycle for P retention. Patients with COPD and in-

progress CKD may be at particular risk and a low P diet and, eventually, P binders, should be implemented when indicated [KDIGO (101)].

Another line of future investigation can be to study whether it is useful to add BMD to the survival discrimination indexes in COPD. BMD is not part of the widely used BODE (body mass index, airflow obstruction, dyspnoea, and exercise capacity) and ADO (age, dyspnoea, and airflow obstruction) indexes in COPD (198), but several comorbidities were included in the most recently described COTE index (COPD specific comorbidity test) (199). The condition of osteoporosis was not included but there was a rather low prevalence of osteoporosis [10% versus the accepted 35% stem from meta-analysis (179)] and the difference of low BMD across survivors and non-survivors was borderline (p=0.07). Because the assessment of comorbidities was based on direct questioning, an underestimation of low BMD and vertebral fractures cannot be discarded. It calls for further investigation to test for a potential role of the inclusion of BMD on survival discrimination indexes of COPD patients.

- ❖ P and coronary artery calcification (CAC)

Our findings from Mendelian Randomization analysis of an increasing yet normal serum P as a causal factor in CAC pathogenesis in the general population of healthy subjects may have importance especially because an increasing - but still normal - P has been associated with the hazard of dying of CV diseases (106), especially in men (85).

Severe hyperphosphatemia is a well-known causative factor for arterial calcification, as extensively shown in advanced CKD (101) and in Mendelian disorders with loss of function mutations in *FGF23* and *KLOTHO* (61,177).

However, in the general context of normophosphatemia, P intake might also be a causative factor for arterial calcification as it has been shown that a P (or calcium) enriched meal intake is immediately followed by an acute increase in portal P and

subsequent nucleation – i.e., an association with calcium to create calciprotein particles (CPPs), recently described to be related to arterial calcification pathogenesis (103). If our findings from the MR study can be replicated and if more prospective studies with varying amounts of P intake would confirm detrimental effects of high P intake on health, this could lead to general measures to decrease P intake, especially in subjects at higher risk for arterial calcification, such as men, smokers and patients with diabetes mellitus.

- ❖ Genetic variants associated with P

Although we were able to increase the variance explained in serum P levels through the identification of 172 new loci and we implemented fine-mapping in the main associated loci, a large amount of research lies ahead, especially in the application of fine-mapping for the totality of loci and posterior gene prioritization.

Of particular high interest will be the implementation of specialized fine-mapping techniques for the MHC region, characterized by recent population-specific high selection pressure, high variability, long-range haplotypes and strong LD (200). Especially because of the strong LD, the precise identification of the causative variants will be a difficult task. The implementation of trans-ethnic meta-analysis (to leverage the differences in LD in this region, especially between populations from European and Asian ancestries) and subsequent trans-ethnic fine-mapping (170) can offer a high probability of identifying the causative variants responsible for the strongest signal not only from our GWAS, but also from GWAS performed in East Asian ancestry populations (165).

The advance we made, with the largest GWAS ever on phosphate levels so far, in explaining more of the variance in P levels also opens up the possibility to apply polygenic risk scores in stratifying subjects in those with, e.g., high, medium, and low phosphate levels. Together with other risk stratification tools, such genetic predisposition information might be useful in disease management but obviously needs further exploration.

Through this thesis, it is our hope to contribute to the acknowledgment of the importance of bone health status and serum P levels (201). In particular, we hope that our GWAS will be a first step for the improvement and deepening of knowledge in P homeostasis by insights in biology and by MR studies on causality of epidemiological associations, and for the identification of future therapies, of potential benefit for a large fraction of the general population.

References

1. **Browner WS, Seeley DG, Vogt TM, Cummings SR**. Non-trauma mortality in elderly women with low bone mineral density. Study of Osteoporotic Fractures Research Group. *Lancet* 1991;338(8763):355-8.

2. **Johansson C, Black D, Johnell O, Oden A, Mellstrom D**. Bone mineral density is a predictor of survival. *Calcif Tissue Int* 1998;63(3):190-6.

3. **van der Klift M, Pols HA, Geleijnse JM, Van Der Kuip DA, Hofman A et al**. Bone mineral density and mortality in elderly men and women: the Rotterdam Study. *Bone* 2002;30(4):643-8.

4. **Jacobsen SJ, Goldberg J, Miles TP, Brody JA, Stiers W et al**. Race and sex differences in mortality following fracture of the hip. *Am J Public Health* 1992;82(8):1147-50.

5. **Center JR, Nguyen TV, Schneider D, Sambrook PN, Eisman JA**. Mortality after all major types of osteoporotic fracture in men and women: an observational study. *Lancet* 1999;353(9156):878-82.

6. **Trivedi DP, Khaw KT**. Bone mineral density at the hip predicts mortality in elderly men. *Osteoporos Int* 2001;12(4):259-65.

7. **Johansson H, Oden A, Kanis J, McCloskey E, Lorentzon M et al**. Low bone mineral density is associated with increased mortality in elderly men: MrOS Sweden. *Osteoporos Int* 2011;22(5):1411-8.

8. **Mussolino ME, Gillum RF.** Low bone mineral density and mortality in men and women: the Third National Health and Nutrition Examination Survey linked mortality file. *Ann Epidemiol* 2008;18(11):847-50.

9. **Mussolino ME, Madans JH, Gillum RF.** Bone mineral density and mortality in women and men: the NHANES I epidemiologic follow-up study. *Ann Epidemiol* 2003;13(10):692-7.

10. **von der Recke P, Hansen MA, Hassager C**. The association between low bone mass at themenopause and cardiovascular mortality. *Am J Med* 1999;106(3):273-8.

11. **Gonzalez-Macias J, Marin F, Vila J, Carrasco E, Benavides P et al**. Relationship between bone quantitative ultrasound and mortality: a prospective

study. *Osteoporos Int* 2009;20(2):257-64.

12. **Qu X, Huang X, Jin F, Wang H, Hao Y et al.** Bone mineral density and all-cause, cardiovascular and stroke mortality: a meta-analysis of prospective cohort studies. *Int J Cardiol* 2013;166(2):385-93.

13. **Persy V, D'Haese P.** Vascular calcification and bone disease: the calcification paradox. *Trends Mol Med* 2009;15(9):405-16.

14. **Schulz E, Arfai K, Liu X, Sayre J, Gilsanz V.** Aortic calcification and the risk of osteoporosis and fractures. *J Clin Endocrinol Metab* 2004;89(9):4246-53.

15. **Aoyagi K, Ross PD, Orloff J, Davis JW, Katagiri H et al**. Low bone density is not associated with aortic calcification. *Calcif Tissue Int* 2001;69(1):20-4.

16. **Hyder JA, Allison MA, Criqui MH, Wright CM**. Association between systemic calcified atherosclerosis and bone density. *Calcif Tissue Int* 2007;80(5):301-6.

17. **Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia JB**. Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. *Osteoporos Int* 2008;19(8):1161-6.

18. **Block GA, Hulbert-Shearon TE, Levin NW, Port FK**. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998;31(4):607-17.

19. **Gorriz JL, Molina P, Cerveron MJ, Vila R, Bover J et al**. Vascular calcification in patients with nondialysis CKD over 3 years. *Clin J Am Soc Nephrol* 2015;10(4):654-66.

20. **Budoff MJ, Achenbach S, Blumenthal RS, Carr JJ, Goldin JG et al.** Assessment of coronary artery disease by cardiac computed tomography: a scientific statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology. *Circulation* 2006;114(16):1761-91.

21. **Veronese N, Stubbs B, Crepaldi G, Solmi M, Cooper C et al**. Relationship Between Low Bone Mineral Density and Fractures With Incident Car-

diovascular Disease: A Systematic Review and Meta-Analysis. *J Bone Miner Res* 2017;32(5):1126-35.

22. **Duval S, Tweedie R**. Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 2000;56(2):455-63.

23. **Lang TF, Guglielmi G, van Kuijk C, De Serio A, Cammisa M et al**. Measurement of bone mineral density at the spine and proximal femur by volumetric quantitative computed tomography and dual-energy X-ray absorptiometry in elderly women with and without vertebral fractures. *Bone* 2002;30(1):247-50.

24. **Kanis JA, McCloskey EV, Johansson H, Oden A, Melton LJ, 3rd et al**. A reference standard for the description of osteoporosis. *Bone* 2008;42(3):467-75.

25. **Campos-Obando N, Castano-Betancourt MC, Oei L, Franco OH, Stricker BH et al**. Bone mineral density and chronic lung disease mortality: the Rotterdam Study. *J Clin Endocrinol Metab* 2014;99(5):1834-42.

26. **Looker AC, Wahner HW, Dunn WL, Calvo MS, Harris TB et al**. Updated data on proximal femur bone mineral levels of US adults. *Osteoporos Int* 1998;8(5):468-89.

27. **van Durme Y, Verhamme KMC, Stijnen T, van Rooij FJA, Van Pottelberge GR et al**. Prevalence, incidence, and lifetime risk for the development of COPD in the elderly: the Rotterdam study. *Chest* 2009;135(2):368-77.

28. **Johnell O, Kanis JA, Oden A, Johansson H, De Laet C et al**. Predictive value of BMD for hip and other fractures. *J Bone Miner Res* 2005;20(7):1185-94.

29. **Pascual-Guardia S, Badenes-Bonet D, Martin-Ontiyuelo C, Zuccarino F, Marin-Corral J et al**. Hospital admissions and mortality in patients with COPD exacerbations and vertebral body compression fractures. *Int J Chron Obstruct Pulmon Dis* 2017;12:1837-45.

30. **Lau E, Ong K, Kurtz S, Schmier J, Edidin A**. Mortality following the diagnosis of a vertebral compression fracture in the Medicare population. *J Bone Joint Surg Am* 2008;90(7):1479-86.

31. **Masala S, Magrini A, Taglieri A, Nano G, Chiaravalloti A et al**. Chronic obstructive pulmonary disease (COPD) patients with osteoporotic vertebral compression fractures (OVCFs): improvement of pulmonary function after percu-

taneous vertebroplasty (VTP). *Eur Radiol* 2014;24(7):1577-85.

32. **Culham EG, Jimenez HA, King CE.** Thoracic kyphosis, rib mobility, and lung volumes in normal women and women with osteoporosis. *Spine* 1994; 19:1250-1255.

33. **Biskobing DM**. COPD and osteoporosis. *Chest* 2002; 121(2): 609-20.

34. **Kim GW, Joo HJ, Park TS, Lee JS, Lee SW et al**. Vertebral compression fracture may increase mortality in male patients with chronic obstructive pulmonary disease. *Int J Tuberc Lung Dis* 2015; 19(5): 603-9.

35. **Looker AC.** Relationship between femur neck bone mineral density and prevalent chronic obstructive pulmonary disease (COPD) or COPD mortality in older non-Hispanic white adults from NHANES III. *Osteoporos Int* 2014;25(3):1043-52.

36. **Ye C, Xu M, Wang S, Jiang S, Chen X et al**. Decreased Bone Mineral Density Is an Independent Predictor for the Development of Atherosclerosis: A Systematic Review and MetaAnalysis. *PLoS One* 2016;11(5):e0154740.

37. **Zhang Y, Feng B**. Systematic review and meta-analysis for the association of bone mineral density and osteoporosis/osteopenia with vascular calcification in women. *Int J Rheum Dis* 2017;20(2):154-60.

38. **Rennenberg RJ, Kessels AG, Schurgers LJ, van Engelshoven JM, de Leeuw PW et al**. Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. *Vasc Health Risk Manag* 2009;5(1):185-97.

39. **Tota-Maharaj R, Joshi PH, Budoff MJ, Whelton S, Zeb I et al**. Usefulness of regional distribution of coronary artery calcium to improve the prediction of all-cause mortality. *Am J Cardiol* 2015;115(9):1229-34.

40. **Ruiz JL, Weinbaum S, Aikawa E, Hutcheson JD**. Zooming in on the genesis of atherosclerotic plaque microcalcifications. *J Physiol* 2016;594(11):2915-27.

41. **Kado DM, Browner WS, Blackwell T, Gore R, Cummings SR**. Rate of bone loss is associated with mortality in older women: a prospective study. *J Bone Miner Res*.2000;15(10):1974-80.

42. **Kiel DP, Kauppila LI, Cupples LA, Hannan MT, O'Donnell CJ et al.** Bone loss and the progression of abdominal aortic calcification over a 25 year

period: the Framingham Heart Study. *Calcif Tissue Int* 2001;68(5):271-6.

43. **Hak AE, Pols HA, van Hemert AM, Hofman A, Witteman JC.** Progression of aortic calcification is associated with metacarpal bone loss during menopause: a population-based longitudinal study. *Arterioscler Thromb Vasc Biol* 2000;20(8):1926-31.

44. **Nakamura S, Ishibashi-Ueda H, Niizuma S, Yoshihara F, Horio T, Kawano Y**. Coronary calcification in patients with chronic kidney disease and coronary artery disease. *Clin J Am Soc Nephrol* 2009;4(12):1892-900.

45. **Andersson P, Rydberg E, Willenheimer R.** Primary hyperparathyroidism and heart disease-a review. *Eur Heart J* 2004;25(20):1776-87.

46. **Campos-Obando N, Kavousi M, Roeters van Lennep JE, Rivadeneira F, Hofman A et al.** Bone health and coronary artery calcification: The Rotterdam Study. *Atherosclerosis* 2015;241(1):278-83.

47. **Osako MK, Nakagami H, Koibuchi N, Shimizu H, Nakagami F et al.** Estrogen inhibits vascular calcification via vascular RANKL system: common mechanism of osteoporosis and vascular calcification. *Circ Res* 2010;107(4):466-75.

48. **Villa-Bellosta R, Millan A, Sorribas V.** Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am J Physiol Cell Physiol* 2011;300(1):C210-20.

49. **Streicher C, Heyny A, Andrukhova O, Haigl B, Slavic S et al.** Estrogen Regulates Bone Turnover by Targeting RANKL Expression in Bone Lining Cells. *Sci Rep* 2017;7(1):6460.

50. **Fitzpatrick LA**. Gender-related differences in the development of atherosclerosis: studies at the cellular level. *Clin Exp Pharmacol Physiol* 1996;23(3):267-9.

51. **Christian RC, Harrington S, Edwards WD, Oberg AL, Fitzpatrick LA**. Estrogen status correlates with the calcium content of coronary atherosclerotic plaques in women. *J Clin Endocrinol Metab* 2002;87(3):1062-7.

52. **Manson JE, Allison MA, Rossouw JE, Carr JJ, Langer RD et al.** Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007;356(25):2591-602.

53. **Hulley S, Grady D, Bush T, Furberg C, Herrington D et al.** Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 1998;280(7):605-13.

54. **Marjoribanks J, Farquhar C, Roberts H, Lethaby A, Lee J.** Long-term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2017;1:CD004143.

55. **Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR et al.** Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003;349(6):523-34.

56. **Albright F BA, Bloomberg E**. Rickets resistant to vitamin D therapy. *Am J Dis Child* 1937;54(3):529-47.

57. **Jagtap VS, Sarathi V, Lila AR, Bandgar T, Menon P et al.** Hypophosphatemic rickets. *Indian J Endocrinol Metab* 2012;16(2):177-82.

58. **Carpenter TO, Imel EA, Holm IA, Jan de Beur SM, Insogna KL.** A clinician's guide to Xlinked hypophosphatemia. *J Bone Miner Res* 2011;26(7):1381-8.

59. **Imel EA, Econs MJ.** Approach to the hypophosphatemic patient. *J Clin Endocrinol Metab* 2012;97(3):696-706.

60. **Ichikawa S, Baujat G, Seyahi A, Garoufali AG, Imel EA et al.** Clinical variability of familial tumoral calcinosis caused by novel GALNT3 mutations. *Am J Med Genet A* 2010;152A(4):896-903.

61. **Ichikawa S, Imel EA, Kreiter ML, Yu X, Mackenzie DS et al.** A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Clin Invest* 2007;117(9):2684-91.

62. **Ketteler M, Block GA, Evenepoel P, Fukagawa M, Herzog CA, McCann L, et al**. Executive summary of the 2017 KDIGO Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD) Guideline Update: what's changed and why it matters. *Kidney Int* 2017;92(1):26-36.

63. **Ritter CS, Slatopolsky E.** Phosphate Toxicity in CKD: The Killer among Us. *Clin J Am Soc Nephrol* 2016;11(6):1088-100.

64. **Pimentel A, Urena-Torres P, Zillikens MC, Bover J, Cohen-Solal M**.

Fractures in patients with CKD-diagnosis, treatment, and prevention: a review by members of the European Calcified Tissue Society and the European Renal Association of Nephrology Dialysis and Transplantation. *Kidney Int* 2017;92(6):1343-55.

65. **Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG et al**. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J Am Soc Nephrol* 2004;15(8):2208-18.

66. **Kwon YE, Choi HY, Kim S, Ryu DR, Oh HJ; ESRD Registry Committee of the Korean Society of Nephrology**. Fracture risk in chronic kidney disease: A Korean population-based cohort study. *Kidney Res Clin Pract* 2019;38(2):220-8.

67. **ADHR Consortium.** Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nat Genet* 2000;26(3):345-8.

68. **Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A et al**. FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *J Clin Invest* 2003;112(5):683-92.

69. **Bonewald LF, Wacker MJ.** FGF23 production by osteocytes. *Pediatr Nephrol* 2013;28(4):563-8.

70. **Felsenfeld AJ, Levine BS, Rodriguez M**. Pathophysiology of Calcium, Phosphorus, and Magnesium Dysregulation in Chronic Kidney Disease. *Semin Dial* 2015;28(6):564-77.

71. **Vervloet M.** Renal and extrarenal effects of fibroblast growth factor 23. *Nat Rev Nephrol* 2019;15(2):109-20.

72. **Campos-Obando N, Koek WNH, Hooker ER, van der Eerden BC, Pols HA et al**. Serum Phosphate Is Associated With Fracture Risk: The Rotterdam Study and MrOS. *J Bone Miner Res* 2017;32(6):1182-93.

73. **Schlenker RA, VonSeggen WW.** The distribution of cortical and trabecular bone mass along the lengths of the radius and ulna and the implications for in vivo bone mass measurements. *Calcif Tissue Res* 1976;20(1):41-52.

74. **Griffin LM, Honig S, Chen C, Saha PK, Regatte R et al.** 7T MRI of distal radius trabecular bone microarchitecture: How trabecular bone quality varies depending on distance from end-ofbone. *J Magn Reson Imaging* 2017;45(3):872-

8.

75. **Vorland CJ, Stremke ER, Moorthi RN, Hill Gallant KM**. Effects of Excessive Dietary Phosphorus Intake on Bone Health. *Curr Osteoporos Rep* 2017;15(5):473-82.

76. **Pinheiro MM, Schuch NJ, Genaro PS, Ciconelli RM, Ferraz MB et al**. N trient intakes related to osteoporotic fractures in men and women--the Brazilian Osteoporosis Study (BRAZOS). *Nutr J* 2009;8:6.

77. **Zhang R, Lu Y, Ye L, Yuan B, Yu S et al**. Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J Bone Miner Res* 2011;26(5):1047-56.

78. **Higgins JP, Thompson SG, Deeks JJ, Altman DG.** Measuring inconsistency in metaanalyses. *BMJ* 2003;327(7414):557-60.

79. **Delgado-Calle J, Arozamena J, Garcia-Renedo R, Garcia-Ibarbia C, Pascual-Carra MA et al**. Osteocyte deficiency in hip fractures. *Calcif Tissue Int* 2011;89(4):327-34.

80. **Qiu S, Rao DS, Palnitkar S, Parfitt AM**. Reduced iliac cancellous osteocyte density in patients with osteoporotic vertebral fracture. *J Bone Miner Res* 2003;18(9):1657-63.

81. **Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J et al.** Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011;79(12):1370-8.

82. **Wang H, Yoshiko Y, Yamamoto R, Minamizaki T, Kozai K, Tanne K, et al.** Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization in vitro. *J Bone Miner Res* 2008;23(6):939-48.

83. **Martin A, David V, Quarles LD.** Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* 2012;92(1):131-55.

84. **Onal M, Carlson AH, Thostenson JD, Benkusky NA, Meyer MB et al**. A Novel Distal Enhancer Mediates Inflammation-, PTH-, and Early Onset Murine Kidney Disease-Induced Expression of the Mouse Fgf23 Gene. *JBMR Plus* 2018;2(1):32-47.

85. **Campos-Obando N, Lahousse L, Brusselle G, Stricker BH, Hofman A**

et al. Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men. *Eur J Epidemiol* 2018;33(9):859-71.

86. **Onufrak SJ, Bellasi A, Cardarelli F, Vaccarino V, Muntner P et al.** Investigation of gender heterogeneity in the associations of serum phosphorus with incident coronary artery disease and all-cause mortality. *Am J Epidemiol* 2009;169(1):67-77.

87. **Bai W, Li J, Liu J.** Serum phosphorus, cardiovascular and all-cause mortality in the general population: A meta-analysis. *Clin Chim Acta* 2016;461:76-82.

88. **Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G; Cholesterol And Recurrent Events Trial Investigators.** Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005;112(17):2627-33.

89. **Onufrak SJ, Bellasi A, Shaw LJ, Herzog CA, Cardarelli F et al**. Phosphorus levels are associated with subclinical atherosclerosis in the general population. *Atherosclerosis* 2008;199(2):424-31.

90. **Ohnishi M, Razzaque MS.** Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. *FASEB J* 2010;24(9):3562-71.

91. **Morishita K, Shirai A, Kubota M, Katakura Y, Nabeshima Y et al.** The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. *J Nutr* 2001;131(12):3182-8.

92. **Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T et al.** Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997;390(6655):45-51.

93. **Suga T, Kurabayashi M, Sando Y, Ohyama Y, Maeno T et al.** Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* 2000;22(1):26-33.

94. **Nakatani T, Sarraj B, Ohnishi M, Densmore MJ, Taguchi T et al.** In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23) -mediated regulation of systemic phosphate homeostasis. *FASEB J* 2009;23(2):433-41.

95. **Ravikumar P, Ye J, Zhang J, Pinch SN, Hu MC et al.** alpha-Klotho

protects against oxidative damage in pulmonary epithelia. *Am J Physiol Lung Cell Mol Physiol* 2014;307(7):L566-75.

96. **Ravikumar P, Li L, Ye J, Shi M, Taniguchi M et al.** alphaKlotho deficiency in acute kidney injury contributes to lung damage. *J Appl Physiol* (1985) 2016;120(7):723-32.

97. **Kuro OM.** The Klotho proteins in health and disease. *Nat Rev Nephrol* 2019;15(1):27-44.

98. **Yao X, Yuan C, Zhang J, Zhou L, Huang M et al**. Klotho: An important protein in the formation and development of emphysema. *European Respiratory Journal* 2012;40(4536).

99. **Gao W, Yuan C, Zhang J, Li L, Yu L et al.** Klotho expression is reduced in COPD airway epithelial cells: effects on inflammation and oxidant injury. *Clin Sci (Lond)* 2015;129(12):1011-23.

100. **Wang C, Liu X, Zhou Y, Li S, Chen Y et al.** New Conclusions Regarding Comparison of Sevelamer and Calcium-Based Phosphate Binders in Coronary-Artery Calcification for Dialysis Patients: A Meta-Analysis of Randomized Controlled Trials. *PLoS One* 2015;10(7):e0133938.

101. **KDIGO**. Clinical Practice Guideline Update for the diagnosis, evaluation, prevention and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney International Supplement* 2017;7(1):1-59.

102. **Shin S, Kim KJ, Chang HJ, Cho I, Kim YJ et al.** Impact of serum calcium and phosphate on coronary atherosclerosis detected by cardiac computed tomography. *Eur Heart J* 2012;33(22):2873-81.

103. **Kuro-o M**. Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. *Nat Rev Nephrol* 2013;9(11):650-60.

104. **Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A et al.** FGF23 induces left ventricular hypertrophy. *J Clin Invest* 2011;121(11):4393-408.

105. **Kuro OM.** A phosphate-centric paradigm for pathophysiology and therapy of chronic kidney disease. *Kidney Int Suppl* (2011) 2013;3(5):420-6.

106. **Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB et al**. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007;167(9):879-85.

107. **Greenland P, Blaha MJ, Budoff MJ, Erbel R, Watson KE**. Coronary Calcium Score and Cardiovascular Risk. *J Am Coll Cardiol* 2018;72(4):434-47.

108. **Imbens G, Angrist JD.** Identification and estimation of local average effects. *Econometrica* 1994; 62(2):467-76.

109. **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.

110. **Kestenbaum B, Glazer NL, Kottgen A, Felix JF, Hwang SJ et al.** Common genetic variants associate with serum phosphorus concentration. *J Am Soc Nephrol* 2010;21(7):1223-32.

111. **Kanai M, Akiyama M, Takahashi A, Matoba N, Momozawa Y et al.** Genetic analysis of quantitative traits in the Japanese population links cell types to complex human diseases. *Nat Genet* 2018;50(3):390-400.

112. **Haarhaus M, Brandenburg V, Kalantar-Zadeh K, Stenvinkel P, Magnusson P.** Alkaline phosphatase: a novel treatment target for cardiovascular disease in CKD. *Nat Rev Nephrol* 2017;13(7):429-42.

113. **Shuto E, Taketani Y, Tanaka R, Harada N, Isshiki M et al.** Dietary phosphorus acutely impairs endothelial function. *J Am Soc Nephrol* 2009;20(7):1504-12.

114. **Antoniucci DM, Yamashita T, Portale AA.** Dietary phosphorus regulates serum fibroblast growth factor-23 concentrations in healthy men. *J Clin Endocrinol Metab* 2006;91(8):3144-9.

115. **Kemi VE, Kärkkäinen MU, Lamberg-Allardt CJ.** High phosphorus intakes acutely and negatively affect Ca and bone metabolism in a dose-dependent manner in healthy young females. *Br J Nutr* 2006;96:545-52.

116. **Calvo MS, Kumar R, Heath H, 3rd.** Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. *J Clin Endocrinol Metab* 1988;66(4):823-9.

117. **Felsenfeld AJ, Rodriguez M.** Phosphorus, regulation of plasma calcium, and secondary hyperparathyroidism: a hypothesis to integrate a historical and modern perspective. *J Am Soc Nephrol* 1999;10(4):878-90.

118. **Miura Y, Iwazu Y, Shiizaki K, Akimoto T, Kotani K et al.** Identification and quantification of plasma calciprotein particles with distinct physical properties in patients with chronic kidney disease. *Sci Rep* 2018;8(1):1256.

119. **Kim KJ, Kim KM, Park KH, Choi HS, Rhee Y et al.** Aortic calcification and bone metabolism: the relationship between aortic calcification, BMD, vertebral fracture, 25- hydroxyvitamin D, and osteocalcin. *Calcif Tissue Int* 2012;91(6):370-8.

120. **Chow JT, Khosla S, Melton LJ, 3rd, Atkinson EJ, Camp JJ et al.** Abdominal aortic calcification, BMD, and bone microstructure: a population-based study. *J Bone Miner Res* 2008;23(10):1601-12.

121. **Farhat GN, Cauley JA, Matthews KA, Newman AB, Johnston J et al.** Volumetric BMD and vascular calcification in middle-aged women: the Study of Women's Health Across the Nation. *J Bone Miner Res* 2006;21(12):1839-46.

122. **Stubbs JR, He N, Idiculla A, Gillihan R, Liu S, David V, et al.** Longitudinal evaluation of FGF23 changes and mineral metabolism abnormalities in a mouse model of chronic kidney disease. *J Bone Miner Res* 2012;27(1):38-46.

123. **Ubaidus S, Li M, Sultana S, de Freitas PH, Oda K et al**. FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. *J Electron Microsc* (Tokyo) 2009;58(6):381-92.

124. **Pereira RC, Juppner H, Azucena-Serrano CE, Yadin O, Salusky IB et al**. Patterns of FGF23, DMP1, and MEPE expression in patients with chronic kidney disease. *Bone* 2009;45(6):1161-8.

125. **Pereira RC, Valta H, Tumber N, Salusky IB, Jalanko H et al**. Altered Osteocyte-Specific Protein Expression in Bone after Childhood Solid Organ Transplantation. *PLoS One* 2015;10(9):e0138156.

126. **Wesseling-Perry K, Makitie RE, Valimaki VV, Laine T, Laine CM et al.** Osteocyte Protein Expression Is Altered in Low-Turnover Osteoporosis Caused by Mutations in WNT1 and PLS3. *J Clin Endocrinol Metab* 2017;102(7):2340-8.

127. **Demer LL, Tintut Y.** Vascular calcification: pathobiology of a multifaceted disease. *Circulation* 2008;117(22):2938-48.

128. **Paloian NJ, Leaf EM, Giachelli CM.** Osteopontin protects against

high phosphate-induced nephrocalcinosis and vascular calcification. *Kidney Int* 2016;89(5):1027-36.

129. **Scialla JJ, Lau WL, Reilly MP, Isakova T, Yang HY et al.** Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. *Kidney Int* 2013;83(6):1159-68.

130. **Nakahara T, Kawai-Kowase K, Matsui H, Sunaga H, Utsugi T et al.** Fibroblast growth factor 23 inhibits osteoblastic gene expression and induces osteoprotegerin in vascular smooth muscle cells. *Atherosclerosis* 2016;253:102-10.

131. **Bots SH, Peters SAE, Woodward M.** Sex differences in coronary heart disease and stroke mortality: a global assessment of the effect of ageing between 1980 and 2010. *BMJ Glob Health* 2017;2(2):e000298.

132. **Haglin L, Lindblad A, Bygren LO**. Hypophosphataemia in the metabolic syndrome. Gender differences in body weight and blood glucose. *Eur J Clin Nutr* 2001;55(6):493-8.

133. **Jorde R, Sundsfjord J, Bonaa KH.** Determinants of serum calcium in men and women. The Tromso Study. *Eur J Epidemiol* 2001;17(12):1117-23.

134. **Palmer SC, Hayen A, Macaskill P, Pellegrini F, Craig JC et al.** Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. *JAMA* 2011;305(11):1119-27.

135. **Sonu Y, Avinash SS, Sreekantha, Arun Kumar K, Malathi M et al.** Effect of Oestrogen on Altering the Serum and Urinary Levels of Calcium, Phosphate and Magnesium in Hysterectomised Women Compared to Natural Menopausal South Indian Women: A Case Control Study. *Indian J Clin Biochem* 2016;31(3):326-31.

136. **Meng J, Ohlsson C, Laughlin GA, Chonchol M, Wassel CL et al.** Associations of estradiol and testosterone with serum phosphorus in older men: the Osteoporotic Fractures in Men study. *Kidney Int* 2010;78(4):415-22.

137. **Komaba H, Fukagawa M.** FGF23-parathyroid interaction: implications in chronic kidney disease. *Kidney Int* 2010;77(4):292-8.

138. **Perel E, Killinger DW.** The interconversion and aromatization of androgens by human adipose tissue. *J Steroid Biochem* 1979;10(6):623-7.

139. **Burris D, Webster R, Sheriff S, Faroqui R, Levi M et al.** Estrogen directly and specifically downregulates NaPi-IIa through the activation of both estrogen receptor isoforms (ERalpha and ERbeta) in rat kidney proximal tubule. *Am J Physiol Renal Physiol* 2015;308(6):F522-34.

140. **Mendel G**. Versuche uber Plflanzenhybriden. Verhandlungen der naturforschenden Vereines in Brunn. *Abhandlungen* 1866:3-47.

141. **Fisher RA.** The Correlation between Relatives on the Supposition of Mendelian Inheritance. *Transactions of the Royal Society of Edinburgh* 1918;52:399-433.

142. **Loh PR, Tucker G, Bulik-Sullivan BK, Vilhjalmsson BJ, Finucane HK et al.** Efficient Bayesian mixed-model analysis increases association power in large cohorts. *Nat Genet* 2015;47(3):284-90.

143. **Zhang Y, Qi G, Park JH, Chatterjee N.** Estimation of complex effect-size distributions using summary-level statistics from genome-wide association studies across 32 complex traits. *Nat Genet* 2018;50(9):1318-26.

144. **Kim SK.** 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* 2018;13(7):e0200785.

145. **Trajanoska K, Rivadeneira F.** The genetic architecture of osteoporosis and fracture risk. *Bone* 2019;126:2-10.

146. **van Dijk, Zillikens MC, Micha D, Riessland M, Marcelis CL et al.** PLS3 mutations in Xlinked osteoporosis with fractures. *N Eng J Med* 2013; 369(16): 1529-36.

147. **Ott J, Wang J, Leal SM.** Genetic linkage analysis in the age of whole-genome sequencing. *Nat Rev Genet* 2015;16(5):275-84.

148. **Nyholt DR.** All LODs are not created equal. *Am J Hum Genet* 2000;67(2):282-8.

149. **Martin A, Liu S, David V, Karydis A, Feng JQ et al.** Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling. *FASEB J* 2011; 25(8): 2551-62.

150. **Campos-Obando N, Oei L, Hoefsloot H, Kiewiet RM, Klaver CC et**

al. Osteoporotic vertebral fractures druing pregnancy: be aware of a potential underlying genetic cause. *J Clin Endocrinol Metab* 2014; 99(4): 1107-11.

151. **de Bakker CMJ, Tseng WJ, Li Y, Zhao H, Altman-Singles AR, Jeong Y et al**. Reproduction differentially affects trabecular bone depending on its mechanical versus metabolic rate. *J Biomech Eng* 2017; 139(11).

152. **Miyamoto T, Miyakoshi K, Sato Y, Kasuga Y, Ikenoue S, Miyamoto K, et al.** Changes in bone metabolic profile associated with pregnancy or lactation. *Sci Rep* 2019; 9(1): 6787.

153. **Cooke-Hubley S, Gao Z, Mugford G, Kaiser SM, Goltzman D, Leslie WD, et al.** Parity and lactation are not associated with incident fragility fractures or radiographic vertebral fractures over 16 years of follow-up: Canadian Multicentre Osteoporosis Study (CaMos). *Arch Osteoporos* 2019; 14(1): 49.

154. **Vajda EG, Bowman BM, Miller SC.** Cancellous and cortical bone mechanical properties and tissue dynamics during pregnancy, lactation, and postlactation in the rat. *Biol Reprod* 2001;65(3):689-95.

155. **Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, et al**. Osteoporosispseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. *Am J Hum Genet* 1996;59(1):146-51.

156. **Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al**. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 2001;107(4):513-23.

157. **Ke J, Harikumar KG, Erice C, Chen C, Gu X, Wang L, et al.** Structure and function of Norrin in assembly and activation of a Frizzled 4-Lrp5/6 complex. *Genes Dev* 2013;27(21):2305-19.

158. **Nikopoulos K, Venselaar H, Collin RW, et al**. Overview of the mutation spectrum in familial exudative vitreoretinopathy and Norrie disease with identification of 21 novel variants in FZD4, LRP5, and NPD. *Hum Mut* 2010; 31: 656-66.

159. **Tuysuz B, Bursali A, Alp Z, Suyugul N, Laine CM, Makitie O**. Osteoporosis-pseudoglioma syndrome: three novel mutations in the LRP5 gene and response to bisphosphonate treatment. *Horm Res Paediatr* 2012; 77:115-20.

160. **Raimondi D, Orlando G, Messens J, Vranken WF.** Investigating the

molecular mechanisms behind uncharacterized cysteine losses from prediction of their oxidation state. *Hum Mutat* 2017; 38:86-94.

161. **Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H.** Complexity of the genotypephenotype correlation in familial exudative vitreoretinopathy with mutations in the LRP5 and/or FZD4 genes. *Hum Mutat* 2005;26(2):104-12.

162. **Bycroft C, Freeman C, Petkova D, Band G, Elliott LT, Sharp K, et al.** The UK Biobank resource with deep phenotyping and genomic data. *Nature* 2018;562(7726):203-9.

163. **Xu et al.** Estimating genome-wide significance for whole-genome sequencing studies. *Genet Epidemiol* 2014; 38(4): 281-90.

164. **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.

165. **Hirata J, Hosomichi K, Sakaue S, Kanai M, Nakaoka H, Ishigaki K, et al.** Genetic and phenotypic landscape of the major histocompatibility complex region in the Japanese population. *Nat Genet* 2019; 51: 470-80.

166. **Bellenguez C, Strange A, Freeman C, Wellcome Trust Case Control Consortium, Donnelly P, Spencer CCA**. A robust clustering algorithm for identifying problematic samples in genomewide association studies. *Bioinformatics* 2012; 28, 134-5.

167. **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* 2017; 101(1): 5-22.

168. **Tucker G, Price AL, Berger B**. Improving the power of GWAS and avoiding confounding from population stratification with PC-Select. *Genetics* 2014; 197: 1045-49.

169. **Johnson R, Shi H, Pasaniuc B, Sankararaman S.** A unifying framework for joint trait analysis under a non-infinitesimal model. *Bioinformatics* 2018; 34(13): 195-201.

170. **Morris AP**. Transethnic meta-analysis of genomewide association studies. *Genet Epidemiol* 2011; 35(8): 809-22.

171. **Kichaev G, Yang WY, Lindstrom S, Hormozdiari F, Eskin E, Price**

AL, et al. Integrating functional data to prioritize causal variants in statistical fine-mapping studies. *PLoS Genetics* 2014: e10014722.

172. **Delgado-Calle J, Bellido T.** Osteocytes and Skeletal Pathophysiology. *Curr Mol Biol Rep* 2015; 1(4): 157-67.

173. **World Health Organization.** (1994). Assessment of fracture risk and its application to screening for postmenopausal osteoporosis : report of a WHO study group [meeting held in Rome from 22 to 25 June 1992]. *World Health Organization.*

174. **Huang X, Jiang Y, Xia W.** FGF23 and Phosphate Wasting Disorders. *Bone Res* 2013;1(2):120-32.

175. **Razzaque MS.** FGF23-mediated regulation of systemic phosphate homeostasis: is Klotho an essential player? *Am J Physiol Renal Physiol* 2009;296(3):F470-6.

176. **Moe OW.** Familial tumoral calcinosis: a valuable vehicle for discovery. *Nephrol Dial Transplant* 2014;29(12):2155-7.

177. **Sprecher E.** Familial tumoral calcinosis: from characterization of a rare phenotype to the pathogenesis of ectopic calcification. *J Invest Dermatol* 2010;130(3):652-60.

178. **International Osteoporosis Foundation.** Broken bones, broken lives: A roadmap to solbe the fragility fracture crisis in Europe. 2018.

179. **Graat-Verboom L, Wouters F, Smeenk FW, van der Borne BE, Lunde R, Spruit MA**. Current status of research on osteoporosis in COPD: a systematic review. *Eur Respir J* 2009; 34(1): 209-18.

180. **Graat-Verboom L, van der Borne BE, Smeenk FW, Spruit MA, Wouters EF.** Osteoporosis in COPD outpatients based on bone mineral density and vertebral fractures. *J Bone Miner Res* 2011; 26(3): 561-8.

181. **Inoue D, Watanabe R, Okazaki R**. COPD and osteoporosis: links, risks and treatment challenges. *Int J Chron Obstruct Pulmon Dis* 2016; 11: 637-48.

182. **Nuti R, Siviero P, Maggi G, Guglielmi G, Caffarelli C, Crepaldi G et al.** Vertebral fractures in patients with COPD: The EOLO Study. *Osteporos Int* 2009; 20(6): 909-98.

183. **Graat-Verboom L, Smeenk FW, van den Borne BE, Spruit MA, Jan-**

sen FH, van Enschot JWT et al. Progression of osteoporosis in patients with COPD: a 3-year follow-up study. *Respir Med* 2012; 106(6): 861-70.

184. **van Dort MJ, Geusen P, Driessen JHM, Romme EAPM, Smeenk FW, Wouters EFM et al.** High imminent vertebral fracture risk in subjects with COPD with a prevalent or incident vertebral fracture. *J Bone Miner Res* 2018; 33(7): 1233-41.

185. **Bolland MJ, Grey AB, Gamble GD, Reid IR**. Effect of osteoporosis treatment on mortality: a meta-analysis. *J Clin Endocrinol Metab* 2010;95(3):1174-81.

186. **Kranenburg G, Bartstra JW, Weijmans M, de Jong PA, Mali WP, Verhaar HJ et al.** Bisphosphonates for cardiovascular risk reduction: a systematic review and meta-analysis. *Atherosclerosis* 2016; 252: 106-15

187. **Bliuc D, Tran T, van Geel T, Adachi JD, Berger C, van den Bergh J, et al.** Mortality risk reduction differs according to bisphosphonate class: a 15-year observational study. *Osteoporos Int* 2019;30(4):817-28.

188. **Reid IR, Horne AM, Mihov B, Stewart A, Garratt E, Basstin S et al.** Effects of zoledronate on cancer, cardiovascular events, and mortality in osteopenic older women. *J Bone Miner Res* 2020; 35(1): 20-27.

189. **Cummings SR, Lui LY, Eastell R, Allen IE.** Association between drug treatments for patents with osteoporosis and overall mortality rates: a meta-analysis. *JAMA Intern Med* 2019; 179(11): 1491-1500.

190. **Bliuc D, Trach T, van Geel T, Adachi J, Berger C, van den Bergh J et al.** Reduced bone loss is associated with reduced mortality risk in subjects xosed to nitrogen bisphosphonates: a mediation analysis. *J Bone Miner Res* 2019; 34(11): 2001-11.

191. **Ueno M, Maeno T, Nishimura S, Ogata F, Masubuchi H, Hara K, et al.** Alendronate inhalation ameliorates elastase-induced pulmonary emphysema in mice by induction of apoptosis of alveolar macrophages. *Nat Commun* 2015;6:6332.

192. **Xu J XW, Su B**. The effects and mechanism of alendronate for COPD animal model. *Respirology* 2017;22:29-30.

193. **Chang AR, Anderson C.** Dietary Phosphorus Intake and the Kidney.

Annu Rev Nutr 2017;37:321-46.

194. **European Food Safety Authority.** Assessment of one published review on health risks associated with phosphate additives in food. *EFSA Journal* 2013;11(1):3444, 27pp.doi:10.2903/j.efsa.2013.3444.

195. **Chang AR, Lazo M, Appel LJ, Gutierrez OM, Grams ME.** High dietary phosphorus intake is associated with all-cause mortality: results from NHANES III. *Am J Clin Nutr* 2014;99(2):320-7.

196. **Varraso R, Chiuve SE, Fung TT, Barr RG, Hu FB, Willett WC,et al.** Alternate Healthy Eating Index 2010 and risk of chronic obstructive pulmonary disease among US women and men: prospective study. *BMJ* 2015;350:h286.

197. **de Batlle J, Mendez M, Romieu I, Balcells E, Benet M, Donaire-Gonzalez D, et al**. Cured meat consumption increases risk of readmission in COPD patients. *Eur Respir J* 2012;40(3):555- 60.

198. **Brusselle G**. Why doesn't reducing exacerbations decrease COPD mortality? *Lancet Respir Med* 2014;2(9):681-3.

199. **Divo M, Cote C, de Torres JP, Casanova C, Marin JM, Pinto-Plata V et al**. Comorbidities and risk of mortality in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;186(2):155-61.

200. **Okada Y, Momozawa Y, Sakaue S, Kanai M, Ishigaki K, Akiyama M et al.** Deep wholegenome sequencing reveals recent selection signatures linked to evolution and disease risk in Japanese. *Nat Commun* 2018; 9: 1631.

201. **Komaba H, Kaludjerovic J, Hu DZ, Nagano K, Amano K, Ide N et al.** Klotho expression in osteocytes regulates bone metabolism and controls bone formation. *Kidney Int* 2017; 92(3): 599-611.

13

Summary and conclusions

Samenvatting en conclusies

Recent technological advances have provided a valuable set of tools in Imagenology and Pathology, among other disciplines, that have induced an unprecedent burst in our knowledge. Bone tissue has been no exception to these advances; in fact, the last decades have witnessed an ever-growing knowledge in bone physiology and bone pathology fields.

As a consequence, several concepts have gradually evolved, such as the old conceptualization of a) bone as solely a weight bearer and b) fractures as the only skeletal adverse effect when bone tissue is compromised. More recently, the notion of the intriguing evolution of bone from calcium-only aragonite to calcium-and-phosphate hydroxyapatite, has highlighted the tight association between bone tissue and calcium (Ca) but, in particular, between bone tissue and serum phosphate levels (P) – although this relationship is still not widely known, or acknowledged.

Precisely, the first part of this thesis emphasizes on the adverse effects associated with both low bone mineral density (low BMD, not necessarily reaching the osteoporosis threshold) and increasing P levels. Most importantly, we found evidence of a detrimental impact on health beyond fractures. Our data, derived mainly from the Rotterdam Study cohorts but also from MrOS USA, made it possible to conclude that several other outcomes are importantly impaired either when bone density decreases or when P levels increase. Some of these associations have been described previously, but some are hereby described in humans for the first time.

Chapter I provides the introductory section of this thesis book. We mention the concept that bone does not solely provides support and locomotion but, instead, we emphasize on the emerging incorporation of bone tissue within several physiologic axes that play an important role in keeping homeostasis. In particular, the discovery of bone cells as the main source of FGF23, currently considered the key regulator of P levels, has established the “bone-kidney axis”, highly relevant in both health and in diseased conditions.

We provide a brief summary of the main findings of the previous research performed between low BMD and all-cause mortality, which has been the most relevant outcome for bone beyond fracture incidence. Despite the fact that several studies found an association between low BMD and mortality, these findings were initially attenuated through the concept that the association was mediated by shared risk factors for both conditions of low BMD and increased risk of death. Nevertheless, the evidence of low BMD increasing mortality independently of confounders became consistent enough to be successfully demonstrated in meta-analyses.

Yet, the specific type of disease underlying this relationship was not clear. Many research efforts were oriented towards the demonstration of an association between low BMD and increased cardiovascular mortality. The reason for this was the finding of a (apparently) paradoxical association between low BMD and arterial calcification (AC), initially termed the "calcification paradox". Despite the well-known increased risk in CVD mortality that AC confers, no consistent relationship has been found between low BMD and CVD mortality, probably reflecting heterogeneity across sexes and different skeletal sites assessed.

Besides exploring whether a low BMD at the femoral neck (FN-BMD) was related to increased mortality in the Rotterdam Study cohorts independently of fractures, we considered of high relevance the identification of the type of disease in which the condition of a low BMD impairs survival. Data from the Rotterdam Study cohorts allowed the demonstration that a low FN-BMD was related to an increase in all-cause mortality, although our findings were restricted to men. The work done to further explore these associations is the topic of the following Chapter.

Chapter 2 presents a prospective research done in 7.834 participants from the Rotterdam Study I and II cohorts (RS-I, RS-II), including 3392 male participants, in whom each decrease in standard deviation (SD) of FN-BMD (i.e., approx. 0.14 g/cm^2) was related to a 9% increase in all-cause mortality risk [(HR: 1.09 (1.04-1.15)].

We moved forward to identify the specific disease (s) underlying this relationship and intriguingly, we found that our results were mostly driven by mortality due to chronic lung diseases, as follows: a) men with osteoporosis at baseline had a hazard of dying from chronic lung disease during the follow-up of 1560% compared to men with normal FN-BMD at baseline; and b) women with osteoporosis at baseline had a hazard of dying from chronic lung disease of 490% compared with women with normal FN-BMD at baseline.

Of note, chronic obstructive pulmonary disease (COPD) was the specific disease driving this association. It must be added that we performed adjustments for all the potential confounders available in our data sets, including body mass index, smoking, vitamin D status and prevalent and incident vertebral fractures. Importantly, COPD as the main driver of the association between low BMD and mortality has also been described in NHANES III, increasing the consistency of our findings.

Remarkably, we found no association between low FN-BMD and cardiovascular disease (CVD) mortality. This finding adds to the inconsistent studies in the literature in this topic. However, there is a clear evidence from meta-analyses that shows that low BMD is associated with arterial calcification (AC), an important risk factor for CVD mortality. Because most published reports have emphasized the relation of BMD with AC measured in the aorta, we aimed to explore whether a similar relation exists between BMD and AC measured in the coronary arteries (CAC). The following Chapter describes our findings.

In Chapter 3, we analyze the first cohort of the Rotterdam Study (RS-I) and test three associations, as follows: a) the cross-sectional relation of FN-BMD with CAC; b) the prospective relation of FN-BMD loss with CAC; and c) the prospective relation of baseline CAC with fracture risk. We found no association between cross-sectional FN-BMD and CAC. Similarly, there was no evidence of CAC influencing fracture risk. But we found that FN-BMD loss was related to CAC in women (not in men), as follows: each 1% increase in FN-BMD loss was

related to increased CAC scores [β: 0.22 (0.06-0.38)].

However, our results were constrained to women with the lowest 17β-estradiol levels and the highest alkaline phosphatase levels, suggesting that a condition of hypo-estrogenism, in association with increased bone turnover, were mediating our results. In line with this, previous research has shown that the bone-derived protein Receptor Activator of Nuclear Factor κβ (RANK) is expressed in human endothelial cells and that a condition of marked hypo-estrogenism induces its activation not only in bone (with subsequent bone mass loss) but also in the vasculature; in the latter it increases the expression of osteogenic genes, such as *ALPL* (gene encoding for alkaline phosphatase).

We conclude from the first two Chapters the following: a) FN-BMD is associated with mortality in men, and this association is mainly explained by COPD mortality; b) there is no evidence of a primary role of FN-BMD (mainly cortical bone) on coronary artery calcification.

We moved in the following Chapters to investigate whether phosphate levels (P) were associated with several health outcomes in our study population, composed mainly of participants without chronic kidney disease (CKD). The first outcome that we tested was bone-related, namely BMD itself and fracture risk. Our findings are detailed in the following Chapter.

Chapter 4 summarizes our findings from both the Rotterdam Study cohorts and MrOS USA. Intriguingly, P was negatively related to lumbar spine BMD (LS-BMD) in men but not in women while FN-BMD was not related to P levels in either sex. We found that each 1 mg/dL higher P increased fracture risk in 52% in men and in 32% in women. However, only men showed a) a relationship between an increasing yet normal P and fracture risk; and b) a consistent relation between P and fracture risk across the entire range of kidney function, including CKD.

We found a suggestion for a sex dimorphism in the skeletal site affected by

increased P, as men displayed increased fracture risk in trabecular-enriched bones, such as wrist and vertebra, while women displayed increased fracture risk only in the humeral bone.

For the general setting, we propose the concept of "osteocyte deficiency" as a potential explanation of our findings, a term coined by JESUS Delgado-Calle to denote the association between fracture risk and decreased *FGF23* expression in trabecular osteocytes. For the CKD setting, it has been shown that marked increase in FGF23 levels triggered by the progression of CKD induces impaired mineralization and 1,25 dihydroxyvitamin D synthesis.

The second outcome that we tested in association with P was mortality. The results are summarized in the following Chapter.

Chapter 5 describes a prospective study between serum P levels assessed at baseline and mortality risk in two cohorts from the Rotterdam Study followed for more than 10 years. P was found to be related to an increase in all-cause mortality risk in men (46% per 1 mg/dL increase in P levels) but not in women. The assessment of cause-specificity replicated previous evidence of P as a risk factor for increased CVD mortality in men [66% per 1 mg/dL increased P (HR: 1.66 (1.29-2.14))]. Intriguingly, we also found that P was related to COPD mortality in men [344% per 1 mg/dL increased P (HR: 4.44 (2.08-9.49))]. These results were not attenuated when we constrained the analysis to normal P.

Therefore, we identified a consistent relationship between P and COPD mortality in humans for the first time, which proved to be of larger magnitude than P and CVD mortality. Remarkably, an association between P and lung emphysema in animal models has been described since the discovery of the *klotho* gene, an important anti-ageing gene. Normal expression of this gene is required for keeping alveolar integrity, and knockout rodents [*fgf23*$^{-/-}$ and *klotho*$^{-/-}$] develop severe emphysema that can be rescued with restoration of normophosphatemia.

The association between P and CVD mortality has been described previously. From a mechanistic perspective, it is known that high P can induce arterial calcification (AC) in the media layer of the vasculature. However, whether normal P can induce AC has been largely unexplored. We aim to explore it in the first cohort of the Rotterdam Study, and the results are summarized in the following Chapter.

The first section of **Chapter 6** describes a consistent phenotypic relationship between P and AC measured in the coronary arteries (CAC). In the second section, we aim to improve causal inference and applied Mendelian Randomization (MR), and our results were consistent with a causal relationship of P in coronary artery calcification, even after excluding participants with prevalent CV disease, CKD, and hyperphosphatemia. The sex-stratified MR analysis showed a more consistent association in men. This result is in line with a substantial amount of evidence from literature that has found that P is related to adverse outcomes in men only. Nevertheless, this result could be also seen as counterintuitive, because postmenopausal women have higher P levels than men and lack the protective effect of high 17β-estradiol on arterial calcification.

For the sex difference, we can only speculate whether the association of P and ionized calcium is playing a role in our findings. It is well acknowledged that both phosphate and calcium (Ca*P product) are needed for AC pathogenesis and normally, an inverse relationship should be expected between them. However, we (and other authors) have found an inverse relationship between Ca and P in women only.

As most of our results displayed a marked sex difference, we aim to explore further whether there was evidence in our data of a difference between serum calcium and phosphate levels and if so, we aim to identify a potential causative mechanism. These results are summarized in the following two Chapters.

Chapter 7 describes that women from the Rotterdam Study displayed higher calcium and phosphate levels than men. This difference was not fully explained

by differences in levels of gonadal steroids, albumin, $1,25(OH)_2D_3$ or alkaline phosphatase. **In Chapter 8** we describe that the sex difference for calcium and phosphate levels was evident only above 45 years of age.

We moved on to the genetic section of this thesis book in order to explore genetic determinants of low BMD and P levels. For low BMD, we aim to identify genes with large effect, causative of Mendelian disorders. For P levels, we aim to identify multiple sites in the human genome through the implementation of genome-wide association study (GWAS).

Chapter 9 describes the clinical picture of a cluster of five families affected by a mutation in *PLS3*, a gene that encodes for plastin and previously unknown to exert a role in bone. Moreover, we identified an X-linked pattern of inheritance, largely unusual for osteoporosis. As expected, men were more severely affected than women, and some of them displayed multiple fractures (~20) by early adulthood. The clinical picture in women was heterogeneous, but we were able to identify that a common PLS3 single nucleotide polymorphism was associated with increased fracture risk in women within the Rotterdam Study cohorts.

Chapter 10 describes the clinical picture of a young woman with amblyopia in one eye since childhood who complained of severe back pain during pregnancy and delivery. Multiple vertebral fractures were identified in the clinical work-up after delivery. We identified a mutation in *LRP5* as the cause of her severe osteoporosis, located within the spectrum of osteoporosis-pseudoglioma/familial exudative vitreoretinopathy.

Chapter 11 describes the results from the analysis of genetic determinants of serum P levels within the United Kingdom Biobank, a large cohort composed of ~500.000 participants. Our analyses, performed in 392.655 subjects, were able to identify 264 independent signals within the genome associated with P at a stringent statistical level. These signals could be clustered in 182 loci of which 172 are new. We could explain 7.62% of the variance of serum P levels. We found

suggestive evidence of sex-difference in three genetic variants. By far, the most powerful signal mapped to the Major Histocompatibility Complex, at 6p21.31.

As next step, we aim for replication in a large Biobank of different ancestry, perform trans-ethnic meta-analyses and Bayesian fine-mapping.

Samenvatting en conclusies

Recente technologische ontwikkelingen hebben geleid tot een waardevolle set hulpmiddelen voor onder andere medische beeldvorming en pathologie, wat tot een ongekende toename van onze kennis heeft geleid. Botweefsel vormt hierop geen uitzondering. Sterker nog, we zijn de afgelopen decennia getuige geweest van continu groeiende kennis op het gebied van botfysiologie en botpathologie.

Als gevolg hiervan zijn verschillende concepten geleidelijk geëvolueerd, zoals de veronderstelling dat a) bot enkel een rol speelt bij de ondersteuning van het lichaam en b) fracturen het enige schadelijke gevolg zijn van aantasting van het botweefsel. Door nieuwe inzichten in de fascinerende evolutie van bot van enkel uit calcium bestaand aragoniet naar het uit calcium en fosfaat bestaande hydroxyapatiet, is de nadruk gelegd op het nauwe verband tussen botweefsel en calcium (Ca), en bovendien op de relatie tussen botweefsel en serum fosfaat (P). Deze relatie is echter nog steeds niet algemeen bekend en erkend.

Juist in het eerste deel van dit proefschrift wordt de nadruk gelegd op de schadelijke effecten van zowel lage botmineraaldichtheid (lage BMD, waarbij niet noodzakelijkerwijs sprake hoeft te zijn van osteoporose) als verhoogde serum P-waarden. We hebben aanwijzingen gevonden dat het schadelijk effect op de gezondheid zich niet beperkt tot fracturen alleen. Met onze data, voornamelijk afkomstig uit cohorten van de Rotterdam Studie maar tevens van MrOS USA, kunnen we concluderen dat verschillende andere uitkomsten worden beïnvloedt wanneer er sprake is van een afname in de botdichtheid of een stijging in de serum P-waarden Sommige van deze associaties zijn eerder beschreven, maar in dit proefschrift worden een aantal associaties voor het eerst bij mensen beschreven.

Hoofdstuk 1 verzorgt het inleidende gedeelte van dit proefschrift. We benoemen het concept dat bot niet slechts ter ondersteuning en voortbeweging dient, maar benadrukken het groeiende besef dat het botweefsel een belangrijke rol speelt bij verschillende fysiologische processen die van belang zijn bij het in evenwicht

houden van homeostase. Zo is door de ontdekking dat botcellen de belangrijkste bron zijn van FGF23 – momenteel beschouwd als de belangrijkste regulator van de P-waarden – de 'bot-nieras' vastgesteld, welke uiterst relevant is onder zowel gezonde als zieke omstandigheden.

We geven een korte samenvatting van de belangrijkste bevindingen van eerder onderzoek dat is uitgevoerd omtrent een lage BMD en mortaliteit, de meest relevante uitkomst voor bot op fractuurincidentie na. In verschillende studies werd een verband gevonden tussen een lage BMD en mortaliteit maar deze bevindingen werden aanvankelijk verklaard door gedeelde risicofactoren voor zowel een lage BMD, alsook een verhoogd risico op overlijden. Niettemin was het bewijs dat bij een lage BMD de mortaliteit toeneemt, onafhankelijk van verstorende variabelen, consistent genoeg om succesvol aangetoond te worden in meta-analyses.

Toch bleek de specifieke soort ziekte die aan deze relatie ten grondslag lag onduidelijk. Er is veel onderzoek verricht om een verband aan te tonen tussen een lage BMD en verhoogde cardiovasculaire mortaliteit. De reden hiervoor was de ontdekking van een (ogenschijnlijk) paradoxaal verband tussen een lage BMD en slagaderverkalking (atherosclerose, AC), aanvankelijk de 'verkalkingsparadox' genoemd. Ondanks het bekende verhoogde risico op HVZ-mortaliteit als gevolg van AC, is er geen consistent verband gevonden tussen een lage BMD en HVZ-mortaliteit, waarschijnlijk het resultaat van heterogeniteit tussen geslachten en de verschillende plaatsen van het skelet die geëvalueerd zijn.

Naast onderzoek naar een relatie tussen een lage BMD bij de femurhals (FN-BMD) en een verhoogde mortaliteit, onafhankelijk van fracturen in de Rotterdam Studie vonden wij het belangrijk om te onderzoeken welke soort ziekte de kans op overleving vermindert bij een lage BMD. Het werk dat is gedaan voor het verkennen van deze associaties, is het onderwerp van het volgende hoofdstuk.

Data verkregen uit de Rotterdam Studie maakte het mogelijk om aan te tonen dat een lage FN-BMD gerelateerd was aan een toename van mortaliteit door alle

oorzaken, al moet hieraan worden toegevoegd dat onze bevindingen zich beperkten tot mannen. **In Hoofdstuk 2** wordt een prospectief onderzoek gepresenteerd, uitgevoerd onder 7.834 deelnemers uit cohort I en II van de Rotterdam Studie (RS-I, RS-II), waaronder 3.392 mannelijke participanten, bij wie iedere afname van de standaarddeviatie (SD) van FN-BMD (ongeveer 0,14g/cm2) gerelateerd bleek te zijn aan een toename van 9% van het mortaliteitsrisico door alle oorzaken [(HR: 1,09 (1,04-1,15)].

We vervolgden het onderzoek met de identificatie van de specifieke ziekte(s) die aan deze relatie ten grondslag liggen en constateerden dat onze resultaten voornamelijk verklaard worden door mortaliteit als gevolg van chronische longziekten, dat wil zeggen: a) mannen met osteoporose in de uitgangssituatie hadden 1560% meer kans om te overlijden aan chronische longziekte tijdens de follow-up dan mannen met een normale FN-BMD in de uitgangssituatie; en b) vrouwen met osteoporose in de uitgangssituatie hadden 490% meer kans om te overlijden aan chronische longziekte dan vrouwen met een normale FN-BMD in de uitgangssituatie.

Opvallend is dat de chronische obstructieve longziekte (COPD) de belangrijkste verklaring is van het verband tussen BMD en mortaliteit. Hieraan moet worden toegevoegd dat we aanpassingen hebben doorgevoerd voor alle mogelijke storende variabelen die in onze datasets beschikbaar zijn, inclusief body-mass index, roken, de vitamine D-status en aanwezige en incidentele wervelfracturen. Ook de NHANES II beschrijft COPD als de belangrijkste oorzaak van het verband tussen een lage BMD en mortaliteit, wat de consistentie van onze bevindingen versterkt.

Opmerkelijk genoeg hebben we geen verband gevonden tussen een lage FN-BMD en mortaliteit door hart- en vaatziekten (HVZ). Deze bevinding draagt bij aan de inconsistente studies in de literatuur omtrent dit onderwerp. Er is echter duidelijk bewijs uit meta-analyses dat een lage BMD gerelateerd is aan slagaderverkalking (atherosclerose, AC), een belangrijke risicofactor voor HVZ-mortaliteit. Omdat in de meeste gepubliceerde rapporten de nadruk wordt gelegd op de relatie tussen

BMD en AC, gemeten in de aorta, wilden we onderzoeken of er een vergelijkbare relatie bestaat tussen BMD en AC, gemeten in de kransslagaders (CAC). Het volgende hoofdstuk beschrijft onze bevindingen.

In Hoofdstuk 3 analyseren we het eerste cohort van de Rotterdam Studie (RS-I) en testen we drie verbanden: a) de cross-sectionele relatie tussen FN-BMD en CAC; b) de prospectieve relatie tussen FN-BMD-afname en CAC; en c) de prospectieve relatie tussen CAC in de uitgangssituatie en het fractuurrisico. We vonden geen verband tussen cross-sectionele FN-BMD en CAC. Er was evenmin bewijs dat CAC het fractuurrisico beïnvloedde. We ontdekten wel dat FN-BMD-afname gerelateerd was aan CAC bij vrouwen maar niet bij mannen: iedere 1% toename in FN-BMD-afname was gerelateerd aan een toename van de CAC-scores [β: 0,22 (0,06-0,38)].

Onze resultaten bleven echter beperkt tot vrouwen met de laagste 17β-estradiolwaarden en de hoogste alkalische fosfatasewaarden, wat suggereert dat een laag oestrogeen gehalte, in combinatie met een verhoogd botmetabolisme, onze resultaten medieerde. Overeenkomstig dit resultaat heeft eerder onderzoek aangetoond dat het van bot afgeleide eiwit Receptor Activator van Nucleaire Factor-kappa B (RANK) tot expressie komt in de menselijke endotheelcellen en dat een laag oestrogeen gehalte zijn activatie niet alleen in bot induceert (met botmassaverlies tot gevolg) maar ook in het vaatstelsel. In het laatste geval verhoogt het de osteogene genexpressie, waaronder het enzym alkalische fosfatase.

Uit de eerste twee hoofdstukken concluderen we het volgende: a) FN-BMD is geassocieerd met mortaliteit onder mannen, en dit verband wordt voornamelijk door COPD-mortaliteit verklaard; b) er is geen bewijs dat FN-BMD (voornamelijk corticaal bot) een primaire rol speelt bij verkalking van de kransslagaders.

In de volgende hoofdstukken gaan we verder met het onderzoeken van het verband tussen serumfosfaatwaarden (P) en verschillende gezondheid-gerelateerde uitkomsten binnen onze onderzoekspopulatie, die voornamelijk bestaat uit

participanten zonder chronisch nierfalen (CKD). De eerste uitkomst die we testten was botgerelateerd, namelijk BMD zelf en het fractuurrisico. Onze bevindingen worden in het volgende hoofdstuk in detail beschreven.

Hoofdstuk 4 biedt een samenvatting van onze bevindingen in zowel de Rotterdam Studie- als MrOS USA. Het viel op dat P negatief gerelateerd was aan de BMD van de lumbale wervelkolom (LS-BMD) bij mannen maar niet bij vrouwen, terwijl FN-BMD duidelijk niet gerelateerd was aan P-waarden in beide geslachten. We vonden dat elke 1 mg/dL toename van P het risico op fracturen verhoogt met 52% bij mannen en met 32% bij vrouwen. Echter, alleen mannen vertoonden a) een verband tussen een toenemende maar normale P-waarde en het fractuurrisico; en b) een consistente relatie tussen P en het fractuurrisico over het gehele bereik van het functioneren van de nieren, inclusief mensen met CKD.

We vonden een suggestief geslachtsverschil in de door verhoogde P-waarden beïnvloede skeletplaats, aangezien mannen een verhoogd fractuurrisico vertoonden in trabeculair verrijkte botten, zoals de pols en de wervels, terwijl vrouwen enkel een verhoogd fractuurrisico vertoonden in het bot van de bovenarm.

Voor het algehele kader stellen we het concept van ‘osteocytdeficiëntie’ voor als een mogelijke verklaring voor onze bevindingen, een term geïntroduceerd door Jesus Delgado-Calle om het verband tussen fractuurrisico en verminderde *FGF23*-expressie in trabeculaire osteocyten aan te duiden. In het kader van CKD is aangetoond dat een duidelijke toename van FGF23-waarden, veroorzaakt door de progressie van CKD, een verstoorde mineralisatie en 1,25-dihydroxyvitamine D-synthese induceert.

De tweede uitkomst die we hebben getest in verband met P is de mortaliteit. De resultaten hiervan zijn samengevat in het volgende hoofdstuk.

In Hoofdstuk 5 wordt een prospectieve studie beschreven tussen serum P-waarden in de uitgangssituatie en het mortaliteitsrisico binnen twee cohorten van de

Rotterdam Studie die meer dan 10 jaar werden gevolgd. P bleek gerelateerd te zijn aan een toename van het mortaliteitsrisico door alle oorzaken onder mannen (46% per 1 mg/dL toename van de P-waarden) maar niet bij vrouwen. Een analyse van verschillende oorzaken van mortaliteit bevestigde de eerdere bevinding dat P een risicofactor is voor verhoogde HVZ-mortaliteit onder mannen [66% per 1 mg/dL verhoogde P (HR: 1,66 (1,29-2,14)]. Interessant is dat P tevens gerelateerd was aan COPD-mortaliteit bij mannen [344% per 1 mg/dL verhoogde P [HR: 4,44 (2,08-9,49)]. Deze resultaten werden niet afgezwakt toen we de analyse beperkten tot mensen met een normale P spiegel.

Hiermee hebben we voor het eerst een consistente relatie aangetoond tussen P en COPD-mortaliteit bij mensen, een relatie die sterker bleek te zijn dan die tussen P en HVZ-mortaliteit. Opmerkelijk is dat een verband tussen P en longemfyseem in diermodellen is beschreven sinds de ontdekking van het *klotho*-gen, een belangrijk anti-verouderingsgen. Normale expressie van dit gen is vereist voor het behouden van de alveolaire integriteit, en knock-out knaagdieren [*fgf23*-/- en *klotho*-/-] ontwikkelen ernstig emfyseem wat gecorrigeerd kan worden door normofosfatemie te herstellen.

Het verband tussen P en HVZ-mortaliteit is eerder beschreven. Vanuit mechanistisch oogpunt is het bekend dat hoge P-waarden aderverkalking (AC) in de middelste laag van de bloedvaten kunnen induceren. Of een normale P-waarde tot AC kan leiden, is echter niet bekend. Wij streven ernaar dit te onderzoeken middels het eerste cohort van de Rotterdam Studie. De resultaten worden samengevat in het volgende hoofdstuk.

In het eerste deel van **Hoofdstuk 6** wordt een consistente fenotypische relatie beschreven tussen P en AC, gemeten in de kransslagaders (CAC). In het tweede deel streven we ernaar om aan te tonen dat er sprake is van een causale relatie en passen we Mendeliaanse randomisatie (MR) toe. Onze resultaten bleken consistent met een causaal verband tussen P en de verkalking van de kransslagader, zelfs na het excluderen van participanten met reeds aanwezige hart- en vaatziekten,

CKD en hyperfosfatemie. De naar geslacht gestratificeerde MR-analyse toonde een consistenter verband bij mannen. Dit resultaat is in overeenstemming met een aanzienlijke hoeveelheid bewijs uit de literatuur die heeft aangetoond dat P alleen bij mannen gerelateerd is aan negatieve uitkomsten. Niettemin kan dit resultaat ook als contra-intuïtief worden beschouwd, omdat postmenopauzale vrouwen hogere P-waarden hebben dan mannen en het beschermende effect van hoge 17β-estradiol op arteriële verkalking bij hen ontbreekt.

Voor het geslachtsverschil kunnen we slechts speculeren of het verband tussen P en geïoniseerd calcium een rol speelt in onze bevindingen. Het wordt algemeen erkend dat zowel fosfaat als calcium (Ca x P-product) nodig zijn voor de ontwikkeling van AC en normaal gesproken zou er een omgekeerd verband tussen beide mogen worden verwacht. Wij (en andere auteurs) hebben echter alleen bij vrouwen een omgekeerde relatie tussen Ca en P gevonden.

Omdat we bij de meeste van onze resultaten een geslachtsverschil observeerden, willen we verder onderzoeken of we aanwijzingen in onze gegevens kunnen vinden die duiden op geslachtsverschillen tussen serum calcium- en fosfaatwaarden en indien dit het geval is willen we een potentieel causaal mechanisme proberen te identificeren. Deze resultaten zijn samengevat in de volgende twee hoofdstukken.

Hoofdstuk 7 beschrijft dat vrouwen uit de Rotterdam Studie hogere calcium- en fosfaatwaarden vertoonden dan mannen. Dit verschil werd niet volledig verklaard door verschillen in waarden van geslachtshormonen, albumine, $1,25(OH)_2D_3$ of alkalische fosfatase. In **Hoofdstuk 8** beschrijven we dat het geslachtsverschil voor calcium- en fosfaatwaarden alleen evident is boven de 45 jaar.

Vervolgens richten we ons op de genetische sectie van dit proefschrift om genetische determinanten van lage BMD- en P-waarden te onderzoeken. Voor een lage BMD willen we genen identificeren met een groot effect die de oorzaak vormen van Mendeliaanse aandoeningen. Voor de hoogte van P-waarden willen we meerdere locaties in het menselijk genoom identificeren middels een

genoomwijde associatiestudie (GWAS).

Hoofdstuk 9 beschrijft het klinische beeld van een cluster van vijf families met een mutatie in *PLS3*, een gen dat codeert voor plastine en waarvan voorheen onbekend was dat het een belangrijke rol speelt met betrekking tot BMD. Bovendien hebben we een X-gebonden overervingspatroon geïdentificeerd, wat zeer ongebruikelijk is voor osteoporose. Zoals verwacht werden mannen veel zwaarder getroffen dan vrouwen, en sommigen van hen vertoonden al meerdere fracturen (~20) op adolescente leeftijd. Het klinische beeld bij vrouwen was heterogeen, maar we konden vaststellen dat een veel voorkomend *PLS3*-enkel-nucleotide polymorfisme geassocieerd is met een verhoogd fractuurrisico bij vrouwen binnen de Rotterdam Studie.

Hoofdstuk 10 beschrijft het klinische beeld van een jonge vrouw met slecht zicht in een oog sinds de jeugd die zich presenteerde met ernstige rugpijn tijdens zwangerschap en bevalling. Na de bevalling bleek bij de medische valuatie sprake van meerdere wervelfracturen. We slaagden erin om een mutatie in LRP5 te identificeren, binnen de context van een diagnose van het spectrum osteoporose-pseudoglioom - familiaire exudatieve vitreoretinopathie, als de oorzaak van haar ernstige osteoporose.

Hoofdstuk 11 beschrijft de resultaten van de analyse van genetische determinanten van serum P-waarden binnen de Biobank van het Verenigd Koninkrijk, een groot cohort bestaande uit ~500.000 deelnemers. We zijn er in geslaagd 264 onafhankelijke signalen in het genoom van 392.655 personen te identificeren die geassocieerd zijn met P-waarden, onder strikte statistische condities. Deze signalen kunnen worden geclusterd in 182 loci waarvan er 172 niet eerder beschreven zijn. Hiermee kan 7,62% van de variatie van serum P-waarden verklaard worden. We vonden suggestief bewijs voor een geslachtsverschil in drie genetische varianten. Verreweg het sterkte signaal werd gevonden ter plaatse van het Major Histocompatibiliteits Complex, op 6p21.31.

In een volgende stap streven we naar replicatie van onze bevindingen in een grote Biobank met mensen van een andere afkomst waarbij we trans-etnische meta-analyses en Bayesiaanse fine-mapping willen ondernemen.

14 Acknowledgements

Publications

ACKNOWLEDGMENTS / AGRADECIMIENTOS

AL SEÑOR DIOS, SUPREMO CREADOR Y REY DEL UNIVERSO: DIOS PADRE, DIOS HIJO JESUCRISTO Y DIOS ESPIRITU SANTO: por su infinita Bondad y su MISERICORDIA sin límites!! Por todos los regalos que me ha dado de manera inmerecida: mi vida, mi bella familia, mi estudio, por hacerme médico y por la magnífica oportunidad de este Doctorado que puso en mis manos hace 10 años. Por restaurarme la salud plena y generosamente justo antes de abrirme las puertas de Rotterdam. Porque la amabilidad y buena voluntad de tantas bellísimas personas que has puesto en mi camino han sido un fiel reflejo de Vuestro Cuidado y Compasión, pese a mis fallos, uno tras otro.. Porque nunca me ha hecho falta nada, ni en la hermosa Patria que me diste, ni en Holanda, ni en el Reino Unido, ni en Japón.. ni en ningún sitio donde he ido. Por permitirme culminar esta etapa de mi vida, sin duda alguna inmerecida!

A LA SANTISIMA VIRGEN DEL CARMEN Y AL GLORIOSO PATRIARCA SAN JOSE, por su ayuda incondicional, por su guía y por su auxilio en momentos difíciles. Por todas las Bendiciones inmerecidas y todas las Oraciones escuchadas y respondidas de una manera generosa y superior a mis limitadas expectativas. Por hacer posible empezar, continuar y culminar este grado académico. Que sirva lo que resta de mi vida para serviros y servir al prójimo, de manera poderosa!

POR TODA LA INTERCESION DE LOS SANTOS Y ANGELES manifestada en protección y ayuda continua.

A mi familia: esa bellísima familia que DIOS me ha regalado, en especial: mis padres, hermanos, cuñados y sobrinos! Motor en el día a día. Fuente continua de energía y motivación. Auxilio y soporte, pese a mis errores y limitaciones. Ejemplo de trabajo, de FE, de entrega, de perseverancia y de caridad. Gracias por levantarme en momentos difíciles, por la ayuda incondicional!

A mis queridos papás: gracias por el fantástico apoyo - desde siempre!! - en mis planes, en mis sueños, en mis ideas.. aunque no fuesen sensatas. Gracias por los consejos de oro y por preocuparse siempre por mí. Por los valores inculcados. Por el afecto, la comprensión, el auxilio, la tolerancia, la inmensa paciencia... Porque siempre han estado conmigo: en los momentos buenos y en los momentos difíciles de este camino académico. Pero, también en los caminos no académicos que he recorrido hasta hoy. ¡Gracias por el apoyo sin medida para terminar mis estudios en Holanda! Gran parte de este título es de hecho de ustedes, mis queridos papás!

A mis hermanos: gracias por ser como son, por ese corazón generoso, por la ayuda sin medida, por la tolerancia, por la actitud siempre dispuesta para apoyarme en todo. *En especial, a Anita, Eric y Miguel*: gracias por la ayuda con la beca para terminar este sueño académico y por confiar en mí!! *Anita*: gracias por tu profundo y continuo apoyo, por estar al pendiente de todos mis asuntos y porque siempre ofreces tu generosa ayuda (a mí y a todos en casa). En síntesis: por ese corazón que tienes, lleno de Caridad y Buena Voluntad.

A mis sobrinitos: gracias por las muestras de cariño y apoyo. Que DIOS les abra Grandes Puertas en sus vidas!

- In the Netherlands

To my Promotors: Professor Zillikens and Professor Uitterlinden.

Dear Prof. Dr. Zillikens: many thanks!!! Finally, and thank ALMIGHTY GOD this step is getting accomplished after 9 years! In the beginning, you kindly offered me a position as PhD student, and patiently waited until i was able to migrate to the Netherlands. You opened the doors of your pretty house to me without even knowing me. You guided me in my new life in Rotterdam and helped me in the transitional phase. You patiently taught me how to avoid my lack of objectiveness and (plenty of) inconsistencies, both in research as in writing tasks.

By your example, i could learn the importance of strong discipline to keep a continuous and deep daily study. You forgave me many mistakes: some of them small, some of them not. But you always offered me your comprehension and understanding. Thanks for trusting me since the early beginning, while barely knowing me. Thanks for trusting that i was going to be able to finish successfully. Your Professorship is more than deserved and indeed, for your students you have been already a Professor since long ago! Thanks for allowing me to accomplish another step in my career: through it, i hope to serve my country and my naaste.

Dear Prof. Dr. Uitterlinden: thanks for allowing me to become part of your wonderful research group! Thanks for all your support and your patience. For all the letters to Costa Rica you kindly and patiently wrote every time i needed them. For your guide for research, for your objectiveness. For your support until finalizing this important step in my life. For your sincere advice – sometimes beyond academic boundaries – and for your concern to solve several obstacles that appeared on the way. Thanks for supporting my plans concerning the UK Biobank. Thanks for all the valuable teachings i learned from you. Apologies for all the times i have disappointed you!

Dear Prof. Dr. WW de Herder: you were the first person to welcome me in the Netherlands. You allowed me to follow the internship in the Endocrinology Department in Erasmus MC in September, 2010. You guided me and introduced me to Prof. Zillikens, who kindly offered me a position as PhD student. Thanks for all your support, your kindness with me and with my parents and your willingness to help. Thanks for all the knowledge i acquired from your vast experience and sharp intelligence. I will always be in debt to you!

Geachte Prof. Pols, Rector Magnificus Erasmus Universiteit 2013-2018: dear Professor, thanks for your valuable input in our group and for sharing your vast knowledge and research skills with us. For all your wise academic advice. Many thanks for your time and good will to listen to my PhD project while being the Rector, despite your extremely busy agenda. Importantly, many thanks for your

kind offer to be the Chair of my PhD Defense! It has been a great Honor for me and my family! We will always be thankful to you.

Dear Dr. JAMJL Janssen: thanks for your help and your continuous concern. Thanks for your interest about my academic performance and wise advice. Finally, i finish the book!

Dear Prof. Dr. van der Lely, Dr. Feldeers & Dr. Neggers: many thanks for the wonderful internship you allowed me to follow! Thanks for all your teachings, for sharing your wonderful experience with me!

A María Carolina Medina: ¡querida amiga, ha sido un gusto compartir contigo estos años! Gracias por tu apoyo, tu sinceridad, tu ejemplo de trabajo fuerte, honrado, de altísima calidad. Gracias por tus valiosos consejos y guía, especialmente académica. Sin duda eres un instrumento valiosísimo en el grupo por tu gran talento e inteligencia, aunados a un profundo sentido de responsabilidad y capacidad para el trabajo arduo. DIOS te ha bendecido con esa familia que estrenaste hace poquito: que EL te siga Bendiciendo y abriendo Grandes Puertas!

Dear Ms Yvonne and Mr Cees: You have always been there for me, since the very beginning! i ill never forget that Saturday of October, 2011, when you waited for me to fill the apartment with plenty of good things! You have shown me a high dose of affection, concern and support! You have not only provided me the support of a family but also have also granted me advice full of Wisdom, which have been necessary to take difficult and important decisions. You have always care that i have all the necessary tools to continue. You have always supported my family as well and welcome them in a generous way. Your support, affection and comprehension have been a clear reflection of the deep MERCY from **ALMIGHTY GOD**, a reflection of his LOVE, a manifestation of HIS CONTINUUM CARE. Apologies my long periods of absence, especially in the last years! Dear Ms Yvonne and Mr Cees: also on behalf of my family, there will never be enough words to express our gratitude .. May **GOD** bless you, always!

Dear Ms Ina, it has been a pleasure to get to know you, already 9 years ago! Thanks for the beautiful apartment! Thanks for your continuous concern, for your affection, for your advice. For taking care of me, especially at difficult times! Apologies my long periods of absence, especially in the last year! Hope to be able to go back in a not-so-far future!

Dear Olgi and Sergio: Olgi, my dear friend: you have always been supportive and concerned about my health, my well-being, my academic performance. You have always offered your great friendship and support in many diverse ways. Your heart is full of honesty and good will. Many thanks for your great friendship and helping hands in difficult times! May **GOD** keep Blessing you and helping you; may **THEOTOKOS** protect you, altijd. Querido Sergio: gracias por tu ayuda, tu gran amistad, y tu buena voluntad. Gracias por estar pendiente. Te deseo un futuro lleno de éxitos y de FE pero especialmente, de la **GRACIA del SEÑOR** para ti y para Olgi. Que **DIOS** les Bendiga esa unión y los colme de salud y alegrías.

A Martha C y Ling: queridas amigas, gracias por todos los años compartidos, por toda la ayuda académica y no académica, por todo el apoyo y las experiencias compartidas!

To Marijn and Joost: dear friends, thanks for your help and support throughout these years! Thanks for your constant patience in addressing my requests, no matter how naïve they could have been… Thanks for your friendship and concern. May **GOD** keep Blessing you and grant you all the dreams you have.

Dear Lies: thanks for your help and support with technical issues. Thanks for your concern and friendship.

Dr. Rivadeneira: gracias por el apoyo durante estos años, por todas las enseñanzas, por todos los valiosos consejos. Este tiempo en Holanda ha sido una experiencia profunda para mí, por haber podido compartir con este maravilloso grupo! Que

DIOS lo siga Bendeciendo, también a su linda familia, y le conceda todos los deseos de su corazón.

Prof. Dr. Franco: gracias por todo el apoyo, querido Profesor. Ha sido una gran pérdida que te fueras de Erasmus; tus estudiantes te extrañamos! Gracias por compartir nuestra **FE**. Que **DIOS** te bendiga siempre, y te recompense el generoso corazón que tienes!

Dear Miss Eline: many thanks for all your help though this years; i will never forget!

Dear Ariadne, Jin, Vivi, Bohar and all of you: thank you! Many **BLESSINGS**

A Johanna: querida amiga, tu amistad ha sido en extremo valiosa para mí! Me has impulsado en momentos difíciles, me has aceptado siempre y me has tendido tu mano. Has sido un soporte importante para mantener nuestra **FE CATOLICA**. Gracias por tu impulso, por tu preocupación genuina por mí!! Gracias por toda tu apoyo para mi viaje a Japón! Gracias por tus Oraciones! Y por tener para mi siempre consejos llenos de la Sabiduría del **ESPIRITU SANTO**. Te extrañaré! Te deseo lo mejor: que **DIOS** te colme de Bendiciones y te abra Grandes Puertas en tu vida! Sigue adelante: eres un gran Instrumento del **SEÑOR**.

To the *MISSIONARIES OF CHARITY* (*Sisters*) from **HOLY MOTHER THERESA**: dear Sisters, thanks so much for allowing me being part of your Daily Holy Mass during such a long time! Thanks for your advice and Prayer! The daily **WORD** and **HOLY COMMUNION** has deeply helped me every day, it has kept my Faith alive! Keep on!! Wish you many **BLESSINGS** from **GOD**! i will miss you!

- In Iowa, USA

A Laurita: querida amiga, gracias por tu amistad que ha perdurado durante

tanto tiempo y por el inmenso favor que me hiciste cuando mi salud estaba comprometida! Sin tu ayuda, mis estudios en Holanda no hubiesen sido posible! Gracias por tus Oraciones, tu continua preocupación y estar al tanto! También de parte de toda mi familia, te queremos mucho y nunca olvidaremos lo que has hecho por mí! Que **DIOS** siempre te bendiga!

Dear Ms. Irene Merical, many thanks for helping me so much while being so sick in Iowa. Your help was crucial to recover my health and being able to fly to Netherlands and start this project! There will never be enough words to thank you!! May the **HOLY FAMILY** bless you much!

Dear Dr. Andrew Nish: many thanks for adopting me as your patient! And for your academic concern in my case. My life was fully re-started, literally, after the stent. I will always thank you!! You have a generous and merciful heart: may **GOD** bless you always!

- In Cambridge, UK

Dear Dr. Luan & Prof. Perry: thanks for your invaluable help, for your support, for your analysis and for supporting my dream of a large-scale GWAS on phosphate levels. Thanks for your patience and kindness!

- In Tokyo, Japan

To the Missionaries of Charity (Brothers) in Nihonzutsumi, Tokyo: thanks for all your help and guide in my first trip to Japan! Specially to Brother Sebastian: thanks for your incredible support, that i will never forget! Thanks also for taking me to **HOLY MASS.** May **ALMIGHTY GOD** bless you much and protect you wherever you go! Dear Brothers, you have a great Mission: keep on !! Many thanks for all!

To Prof Terao (RIKEN Center for Integrative Medical Sciences/BioBank Japan):

dear Professor, many thanks for your kindness and your acceptance to cooperate with us in the phosphate GWAS project in order to replicate our findings from UK Biobank in BioBank Japan! Looking forward for a great collaboration to make of this a big project!! May **GOD** bless you.

➢ In **SAN JOSÉ**, Costa Rica

Al Dr. Gei Guardia (qdDg): tus estudiantes disfrutamos tu vasto conocimiento, tus magníficas enseñanzas y tu exquisita agudeza clínica, que, aunado a una profunda humildad, caridad y buena voluntad, hicieron una persona única e irrepetible de la cual obtuve un aprendizaje muy valioso que sobrepasó la esfera académica para adentrarse en el humanismo, en la **FE**, en la generosidad y en la búsqueda genuina del bien del prójimo; lista no *exhaustiva* de las características que hicieron tu alma muy sublime. Gracias, estimado Dr. Gei, por impulsarme a entrar en el mundo del *linkage* y del *lod score*, de los trastornos **Mendelianos** y de la recombinación, y gracias por haber dirigido mi tesis de graduación de Endocrinología con tanta sabiduría. Gracias por preocuparte siempre por la información integral de tus pupilos. Tus pacientes y tus estudiantes te extrañamos, y lo seguiremos haciendo por mucho tiempo!

Al Dr. Ponchner (qdDg): tu ayuda desde el inicio de mis estudios en Holanda fue imprescindible. Siempre me encontré con tu apoyo incondicional pese a los obstáculos que se presentaron. Gracias por creer en mi propuesta de estudios, por impulsarme tanto con palabras alentadoras como con acciones concretas. Lamentablemente, no puedo por ahora compartir la alegría de ver culminado este camino en el que tanto me ayudaste, pero confío en que estés disfrutando de **DIOS** y contemplando su **ROSTRO**.

Al Dr. Montero, Director General, por su apoyo sin medida desde el 2010, cuando se abrían las puertas de Holanda por primera vez. Gracias, estimado Dr, por su aval para los permisos y extensiones, por su comprensión, su apoyo continuo y su paciencia! Gracias por la buena voluntad y generosidad con la que siempre ha

acogido mis múltiples peticiones de favores y prórrogas. Que **DIOS** lo bendiga!

Al Dr. Julián Peña V: gracias por apoyarme profundamente con la parte final de mi tesis ! Gracias por todas las gestiones pertinentes, por la paciencia y buena voluntad!

A la Dra. Villalta Bonilla, Gerente Médico de la CCSS (2012-2018) y a los miembros de la Junta Directiva de la Caja Costarricense de Seguro Social, por su aval para finalizar mis estudios y para las prórrogas.

Al Dr. Esquivel Sánchez, Director Ejecutivo del CENDEISSS, por su gran apoyo para que pudiese continuar mis estudios fuera del país, ayuda que se ha prolongado durante el tiempo y que ha sido fundamental para el buen suceso de este tesis. Querido Dr, siempre le estaré agradecido!

A la Licda. Venegas, Licda Chacón y personal del CENDEISSS: gracias por toda la ayuda que generosa y continuamente me han brindado para poder culminar esta etapa académica de mi vida! Que **DIOS** los bendiga!

A mis colegas y compañeros de Endocrinología, Hospital México: gracias por todo el apoyo, ayuda y comprensión durante estos años! En especial:

- *A mi querida Giselle:* por tu amistad sincera, tu apoyo incondicional, la mística para atender los pacientes y tu gran apoyo durante mis tiempos de ausencia, también para mis papás! Tu ausencia se sentirá de manera profunda en el Servicio.
- A *Mary y Zulema*: gracias por toda la ayuda! En especial con mis papás en mis momentos de ausencia!
- al *Dr. Sibaja*, gracias Dr, por aceptar mi propuesta y ser parte de nuestro grupo! Gracias por tomar el cuidado y control médico de mis pacientitos!
- Al *Dr. José Peña:* gracias Jefe, por tu apoyo y comprensión para poder terminar este sueño académico!
- Y a la *Dra. Yung Lí*: gracias por impulsarme a iniciar la tesis de

Endocrinología en la querida familia con NEM1 en Nandayure, a raíz de la cual se realizó no sólo la investigación genética basada en ligamiento, sino que inicié el camino hacia Rotterdam. Gracias también por el libro de Uma, que me abrió el interés en la estadística Bayesiana – que al día de hoy se ha transformado en una herramienta más fuerte que la estadística frecuentista para la identificación de variantes en el genoma humano asociados a rasgos & enfermedades y para la valoración de *causalidad* a través del mapeo fino.

A mis Profesores de la Universidad de Costa Rica: la *Dra. Henriette Raventós y al Señor Reynaldo Pereira*, por apoyarme con su talento, esfuerzo y buena voluntad para hacer realidad el proyecto de ligamiento en NEM1 - que inicialmente parecía sólo un sueño; y al *Dr. Eric Fuchs Castillo*, por sus maravillosas clases de Genética Cuantitativa, por cultivar siempre el espíritu de investigación en sus estudiantes, y por iniciarnos en los temas de verosimilitud y MCMC.

Al Dr. Edgardo Ramos R: gracias por la valiosa capacitación en Linux y tu ayuda para lograr correr scripts! Gracias por tu buena voluntad y por estar siempre dispuesto a ayudarme!

Al Dr. Randall Cabrera, Dr. Ríos Marín, Dr. Andrés Arley y Dr. Rolando Mora: gracias por estar al pendiente de mis papás y ayudarlos cuando yo me encontraba tan lejos!!! Queridos colegas: que **DIOS** se los recompense de manera abundante!

A Sílvia, mi querida amiga, por tu amistad de tantos años, tu soporte, consejo y preocupación! Gracias por estar siempre ahí!

A mis queridas amigas Katherine y Mariana: gracias por la amistad incondicional pese a mis largas ausencias, por tu disponibilidad a ayudarme en Linux y tu soporte a través de los años.

A doña Isabel Montero: gracias por toda tu ayuda!

A Marta Aguilar: gracias por tu invaluable apoyo para montar esta tesis, desde su portada hasta el último capítulo!! Gracias por todas las correcciones, por todo tu esfuerzo para mejorarla, por todo el empeño y la preocupación para sacarla a tiempo. Por dejar tus propias labores para asumir esta tarea como tuya también. Por tu inmensa paciencia, tu mística, tu buena voluntad y generosidad, que han hecho posible sacar adelante este proyecto y transformar capítulos aislados e ideas sueltas en un proyecto concreto, con formato de libro, propio de una tesis. Gracias por ayudarme en este momento crítico, con generosidad.

Beyond boundaries: to the Priests - Mas allá de las fronteras: a los Sacerdotes
Last, but certainly not least!! To all the Priests here and abroad that have nourished my Faith, increased my devotion, pray for Special Intentions, provided advice full of Wisdom from Above! For all your Prayers, your good will to help me, your patience to listen to me. Thanks for providing the SACRAMENT of reconciliation, for supporting me when most needed, for nourishing my Faith. *A los Sacerdotes*: Padre Herrera, Padre Abarca, Padre Guido, Father Jan, Father Theo, Father van den Hende (Bishop, Rotterdam), Father Gouw, Father de Boer (The Hague), Padre Rafael de Ojeda (Amsterdam); y a todos los demás Sacerdotes: adelante! Ustedes tienen la Misión más Sublime de todas: el hacer posible que podamos cumplir con el pilar de nuestra FE:

"**JESUS** answered them: I am the bread of life. In all truth I tell you, if you do not eat the flesh of the SON of man and drink his blood, you have no life in you. Anyone who does eat my flesh and drink my blood has eternal life, and I shall raise that person up on the last day."
[**HOLY BIBLE**, ST. JOHN (Gospel) 6:35,53,54; New Jerusalem]

"Les dijo **JESUS: YO** soy el Pan de la Vida. En verdad, en verdad os digo: si no coméis la carne del **HIJO** del hombre, y no bebéis su sangre, no tenéis vida en vosotros. El que come mi carne y bebe mi sangre, tiene vida eterna, y **YO** le resucitaré el último día."
[**SANTA BIBLIA**, SN. JUAN (EVANGELIO) 6:35,53,54; Nueva Jerusalem]

PORTFOLIO

Master of Science in Health Sciences. Specialisation: Genetic Epidemiology	2011 - 2013

Oral Presentations

Bone mineral density and chronic lung disease mortality: the Rotterdam Study	ASBMR, Oct 2012
Bone health and coronary artery calcification: the Rotterdam Study	NVCB, Nov 2012
Serum phosphate levels are related to all-cause, cardiovascular and COPD mortality in men	Endocrine Society Meeting, April 2016
Serum phosphate and coronary calcification in the general population: a Mendelian Randomization study	NVCB, Nov 2018

List of Publications

1. **Oei L, Campos-Obando N, Dehghan A, Oei EH, Stolk L, van Meurs JB, Hofman A, Uitterlinden AG, Franco OH, Zillikens MC, Rivadeneira F**. Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study. *Osteoporos Int* 2014; 25(4): 1247-54.
2. **de Jonge EA, Kiefte-de Jong JC, Campos-Obando N, Booij L, Franco OH, Hofman A, Uitterlinden AG, Rivadeneira F, Zillikens MC**. Dietary vitamin A intake and bone health in the elderly: the Rotterdam Study. *Eur J Clin Nutr* 2015; 69(12): 1360-8.
3. **Manousaki D, Dudding T, Haworth S, Hsu YH, Liu CT, Medina-Gómez C, Voortman T, van der Velde N, Melhus H, Robinson-Cohen C, Cousminer DL, Nethander M, Vandenput L, Noordam R, Forgetta V, Greenwood CMT, Biggs ML, Psaty BM, Rotter JI, Zemel BS, Mitchell JA, Taylor B, Lorentzon M, Karlsson M, Jaddoe VVW, Tiemeier H, Campos-Obando N, Franco OH, Utterlinden AG, Broer L, van Schoor NM, Ham AC, Ikram MA, Karasik D, de Mutsert R, Rosendaal FR, den Heijer M, Wang TJ, Lind L, Orwoll ES, Mook-Kanamori DO, Michaëlsson K, Kestenbaum B, Ohlsson C, Mellström D, de Groot LCPGM, Grant SFA, Kiel DP, Zillikens MC, Rivadeneira F, Sawcer S, Timpson NJ, Richards JB**. Low-frequency synonymous coding variation in CYP2R1 has large effects on vitamin D levels and risk of multiple sclerosis. *Am J Hum Genet* 2017; 101(2): 227-38.
4. **Chen J, van der Duin D, Campos-Obando N, Ikram MA, Nijsten TEC, Uitterlinden AG, Zillikens MC**. Serum 25-hydroxyvitamina D3 is associated with advanced glycation end products (AGEs) as skin autofluorescence: The Rotterdam Study. *Eur J Epidemiol* 2019; 34(1): 67-77.
5. **Karasik D, Zillikens MC, Hsu YH, Aghdassi A, Akesson K, Amin N, Barroso I, Bennett DA, Bertram L, Bochud M, Borecki IB, Broer L, Buchman AS, Byberg L, Campbell H, Campos-Obando N, Cauley JA, Cawthon PM, Chambers JC, Chen Z, Cho NH, Choi HJ, Chou WC, Cummings SR, de Groot LCPGM, De Jager PL, Demuth I, Diatchenko L, Econs MJ, Eiriksdottir G, Enneman AW, Eriksson J, Eriksson JG, Estrada**

K, Evans DS, Feitosa MF, Fu M, Gieger C, Grallert H, Gudnason V, Lenore LJ, Hayward C, Hofman A, Homuth G, Huffman KM, Husted LB, Illig T, Ingelsson E, Ittermann T, Jansson JO, Johnson T, Biffar R, Jordan JM, Jula A, Karlsson M, Khaw KT, Kilpeläinen TO, Klopp N, Kloth JSL, Koller DL, Kooner JS, Kraus WE, Kritchevsky S, Kutalik Z, Kuulasmaa T, Kuusisto J, Laakso M, Lahti J, Lang T, Langdahl BL, Lerch MM, Lewis JR, Lill C, Lind L, Lindgren C, Liu Y, Livshits G, Ljunggren Ö, Loos RJF, Lorentzon M, Luan J, Luben RN, Malkin I, McGuigan FE, Medina-Gomez C, Meitinger T, Melhus H, Mellström D, Michaëlsson K, Mitchell BD, Morris AP, Mosekilde L, Nethander M, Newman AB, O'Connell JR, Oostra BA, Orwoll ES, Palotie A, Peacock M, Perola M, Peters A, Prince RL, Psaty BM, Räikkönen K, Ralston SH, Ripatti S, Rivadeneira F, Robbins JA, Rotter JI, Rudan I, Salomaa V, Satterfield S, Schipf S, Shin CS, Smith AV, Smith SB, Soranzo N, Spector TD, Stancáková A, Stefansson K, Steinhagen-Thiessen E, Stolk L, Streeten EA, Styrkarsdottir U, Swart KMA, Thompson P, Thomson CA, Thorleifsson G, Thorsteinsdottir U, Tikkanen E, Tranah GJ, Uitterlinden AG, van Duijn CM, van Schoor NM, Vandenput L, Vollenweider P, Völzke H, Wactawski-Wende J, Walker M, J Wareham N, Waterworth D, Weedon MN, Wichmann HE, Widen E, Williams FMK, Wilson JF, Wright NC, Yerges-Armstrong LM, Yu L, Zhang W, Zhao JH, Zhou Y, Nielson CM, Harris TB, Demissie S, Kiel DP, Ohlsson C. Disentangling the genetics of lean mass. *Am J Clin Nutr* 2019; 109(2): 276-87.