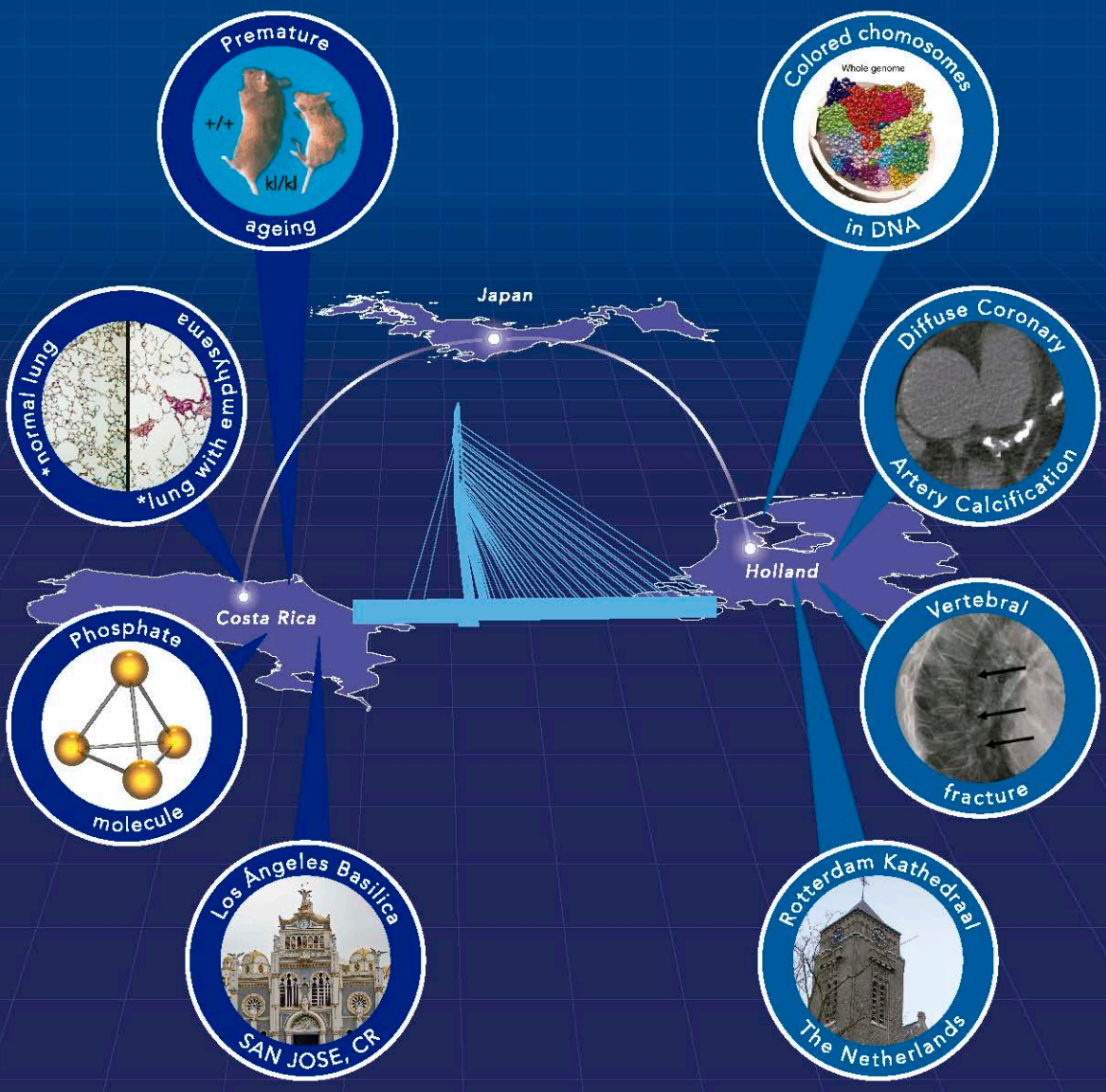
BONE & PHOSPHATE
IN RELATION TO HEALTH, SURVIVAL AND GENETIC FACTORS



natalia campos obando

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

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

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

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natalia campos obando

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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

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PART III.

Genetic studies in relation to bone and phosphate

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.

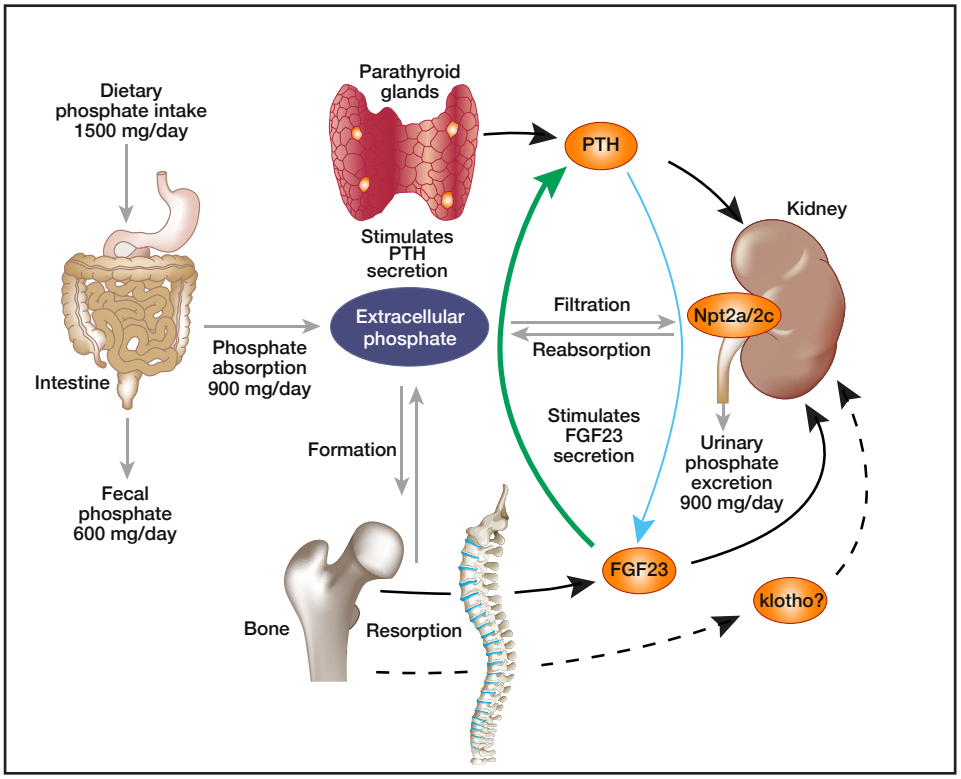

Figure 1. Serum phosphate levels homeostasis (modified from (26)). Continuous arrows show established axes; the intermittent arrows show a suggested axis; the red arrow shows negative homeostatic control. (Reproduced with permission from Copyright Clearance Center, Elsevier. Source (26))

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?*

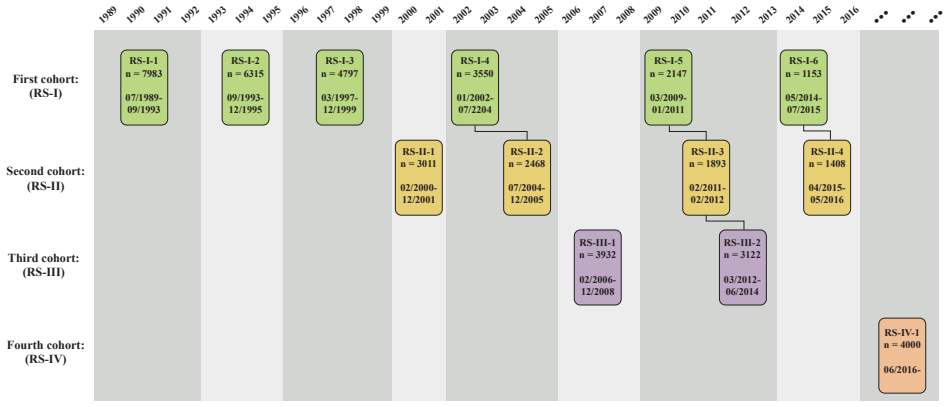

Figure 2. Schematic representation of the Rotterdam Study cohorts, including the baseline visit and the subsequent follow-up examination cycles.

❖ 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.

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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

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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).

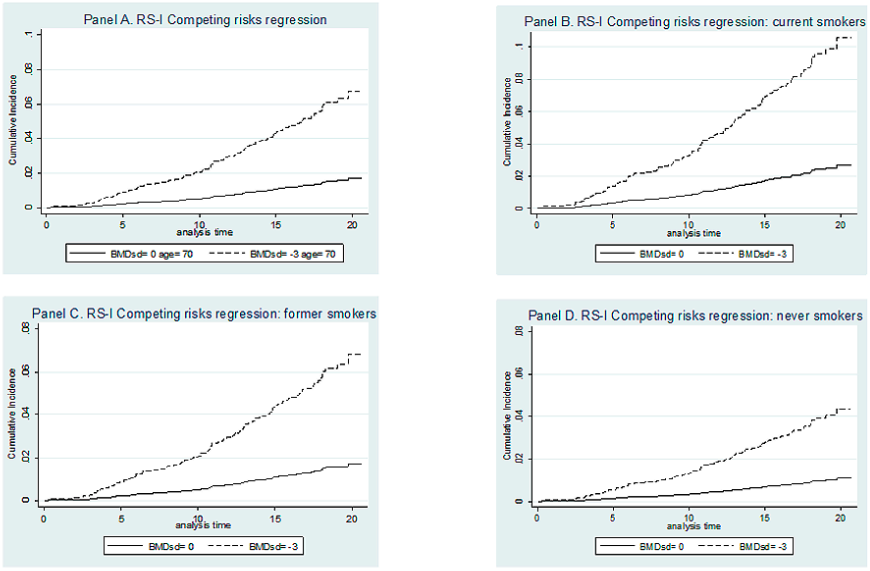

Figure 1. A, CIC for chronic lung disease mortality according to baseline BMD expressed in SD values (BMDsd), adjusted for gender, BMI, lower limb disability and smoking, for participants of median age of RS-I cohort. B-D, CIC according to baseline smoking status: current smokers (B), former smokers (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.

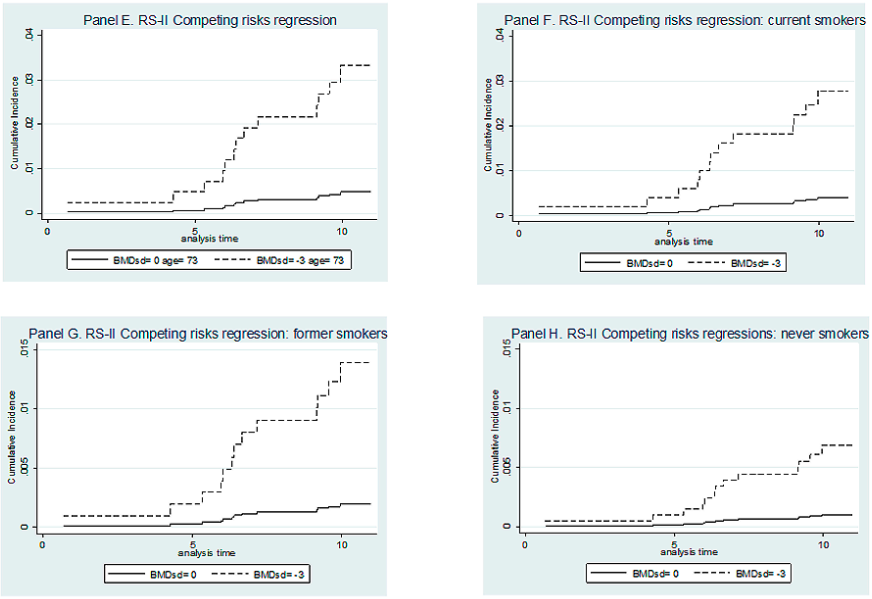

Figure 1. Continued.

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.

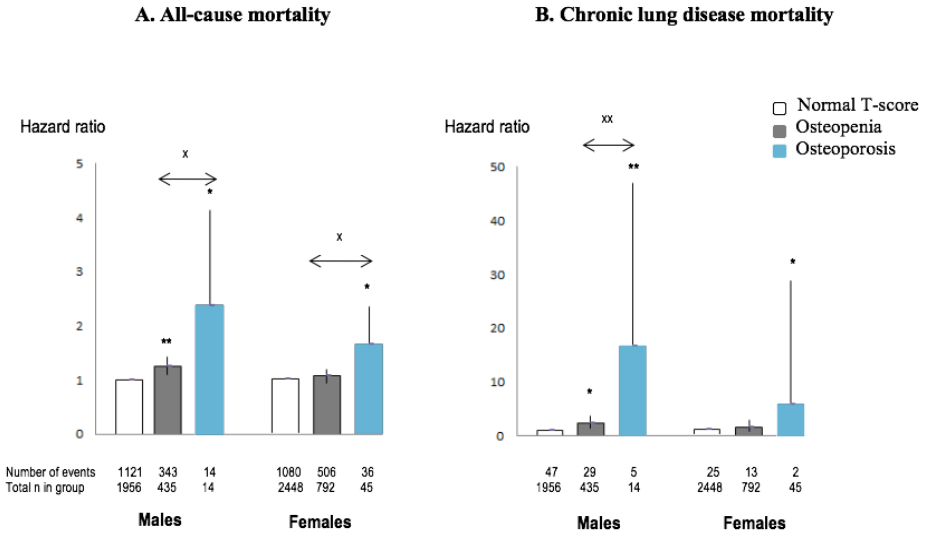

Figure 2. All-cause (A) and chronic lung disease (B) mortality HRs for participants from RS-I, according to T-score, adjusted for age, BMI, and smoking status. *, *P*< .05; **, *P*< .001 compared to normal T-score. x, *P*< .05; xx *P*< .001 osteoporosis compared to osteopenia.

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.

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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

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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.

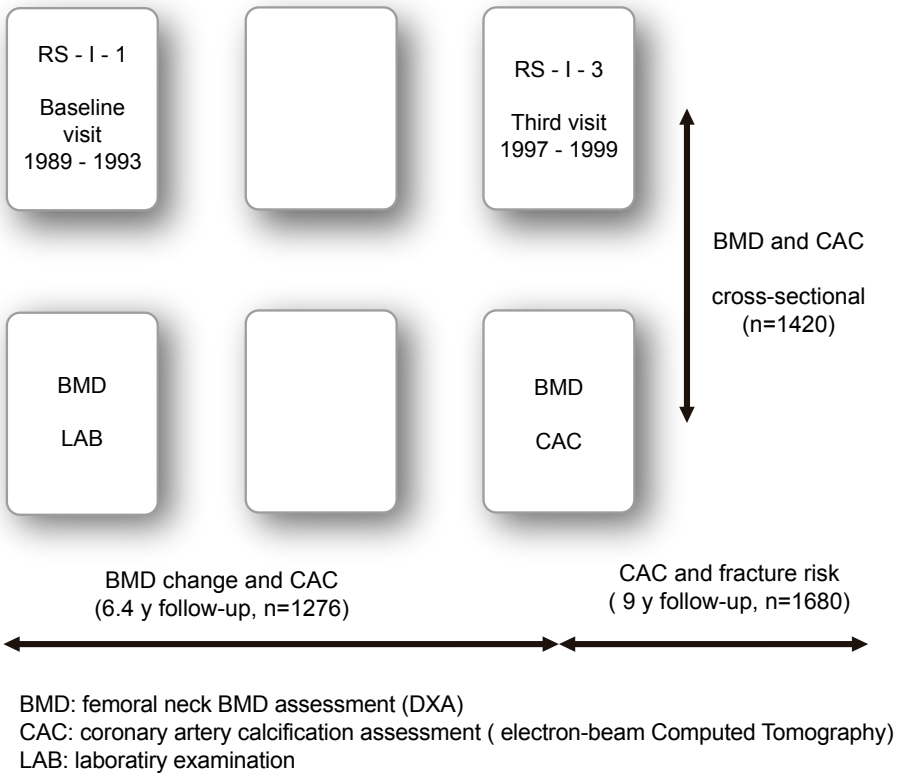

Figure 1. Flowchart for time line, design and sample size for the analyses

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.

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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.

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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

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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

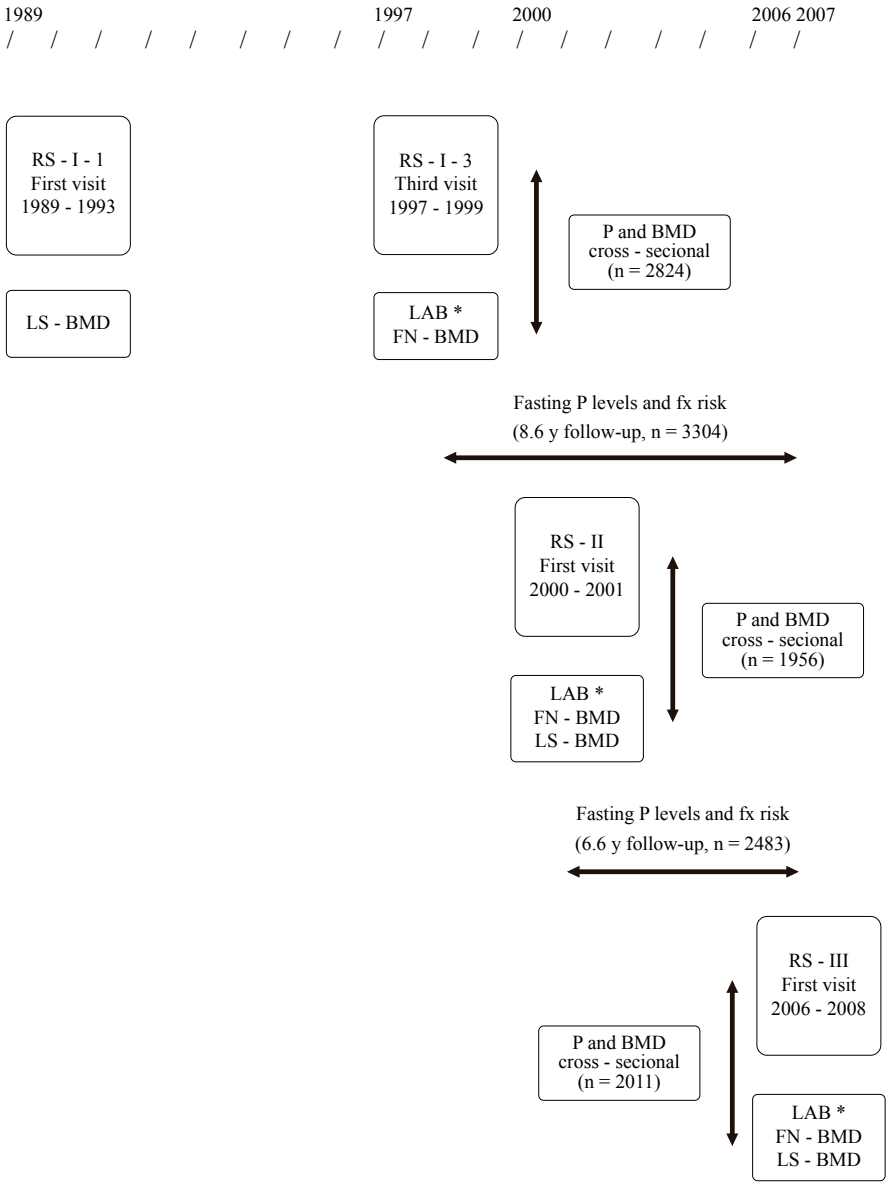

Figure 1. Flowchart for time line, design, and sample sizes for the analyses of the Rotterdam Study cohorts. LAB* includes fasting phosphate levels. FN-BMD = femoral neck BMD; LS-BMD = lumbar spine BMD; P = fasting phosphate levels; Fx risk = fracture risk.

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.

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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

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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].

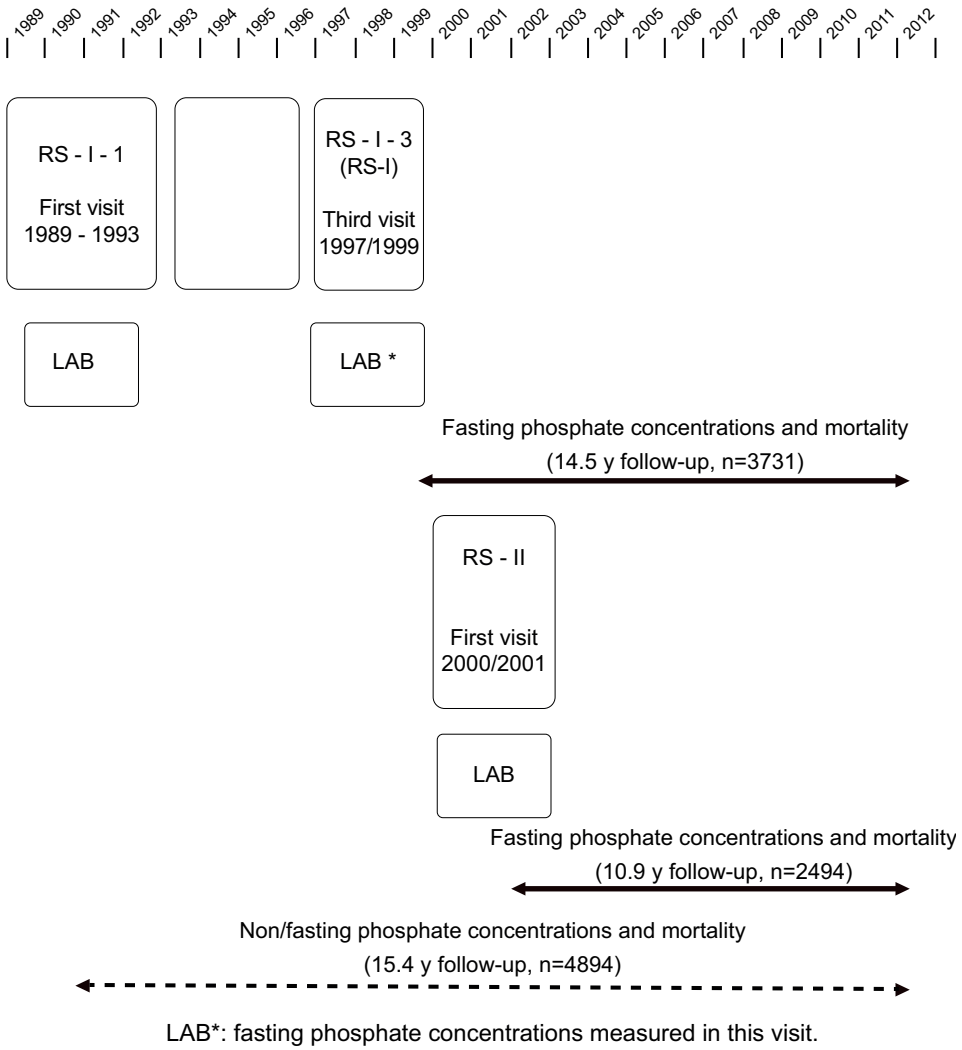

Figure 1. Flowchart for time line, design and sample size for the analyses

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

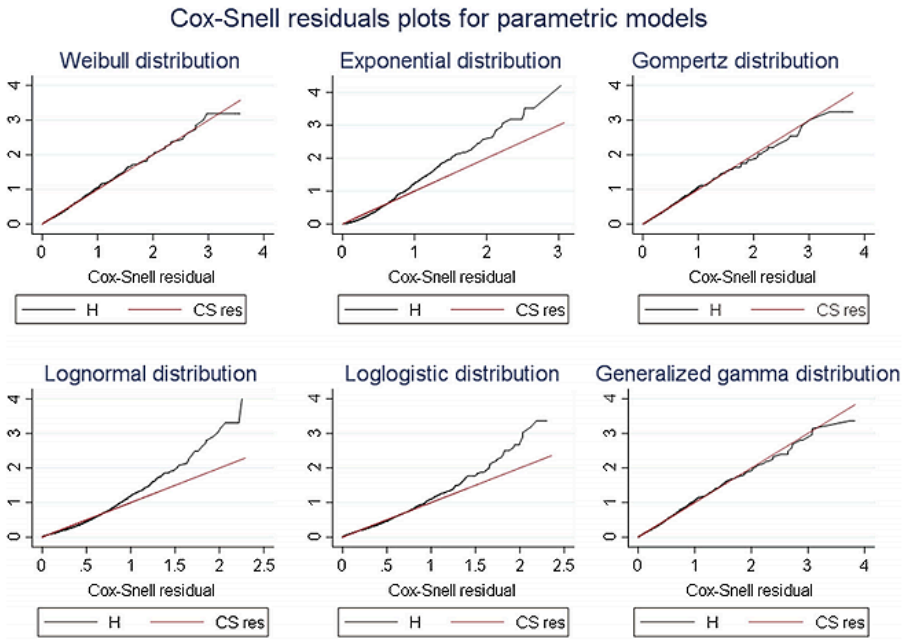

Figure 2. Cox-Snell residuals plot for parametric models in the association between serum phosphate levels and all-cause mortality in men

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.

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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.

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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

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

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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

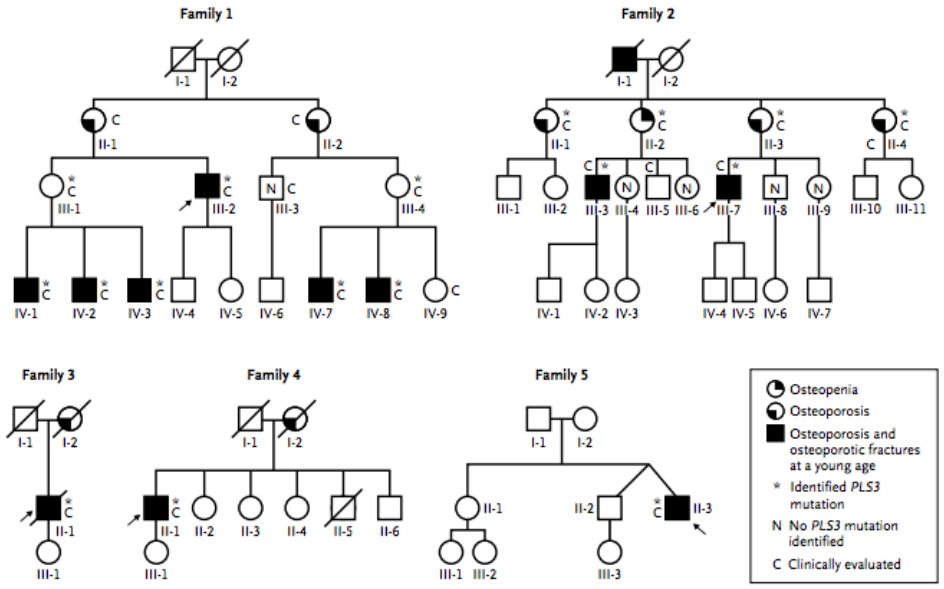
4.II-1); and a frameshift variant, c.1647delC;p.(Ser550Alafs*9), in exon 15 (in Patient 5.II-3). To our knowledge, none of these variants are described in current databases of human sequence variants: data from the 1000 Genomes Project, the Single Nucleotide Polymorphism database (dbSNP, build 137), 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.

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7

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

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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.

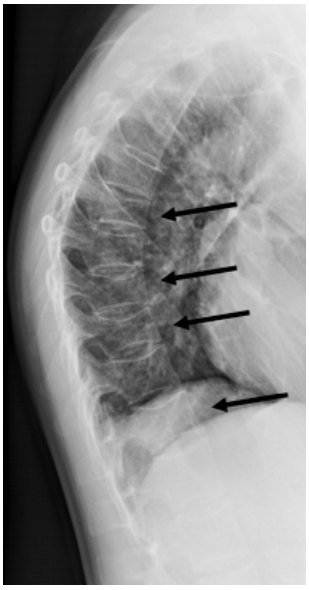

Figure 1. Lateral spinal x-ray image of the index patient. The black arrows show end-plate compressions of thoracic vertebrae at T7, T9, T10, and T12.

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.

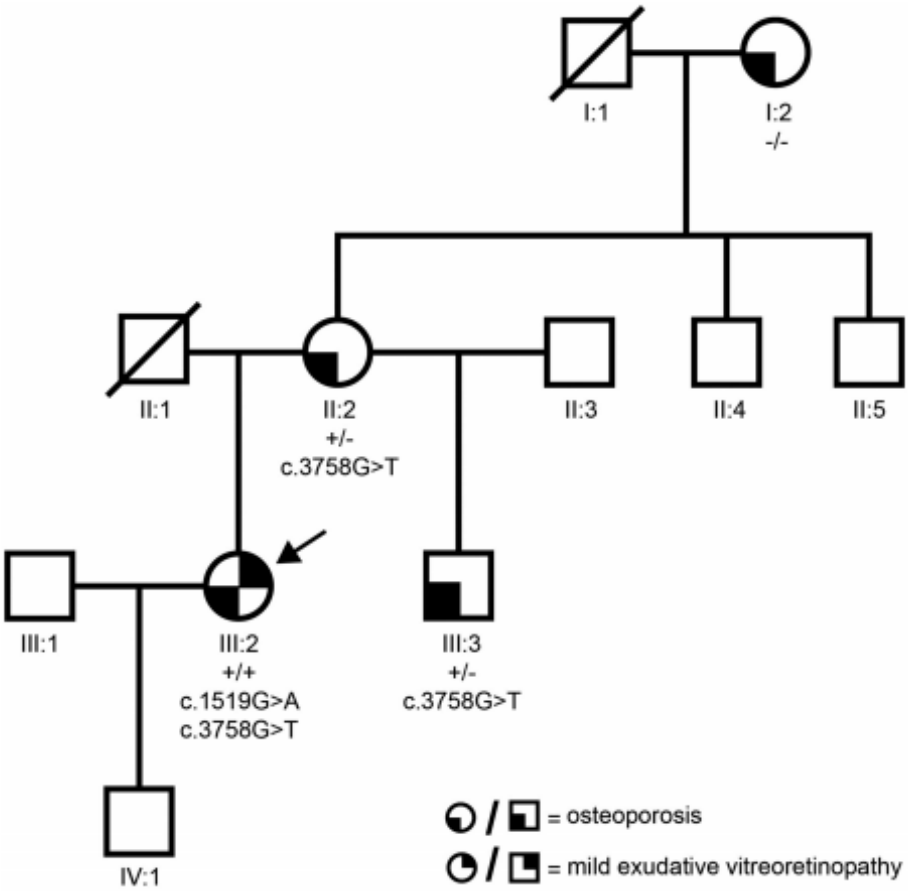

Figure 2. Pedigree and genotypes of the family. Index patient (III:2) is indicated with an arrow. She is compound heterozygous for the c.1519G>A and the c.3758G>T mutations. Subjects II:2 and III:3 are carriers of the c. 3758G>T mutation. Black squares within cicles and square represent low BMD (below 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.

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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]

8 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?

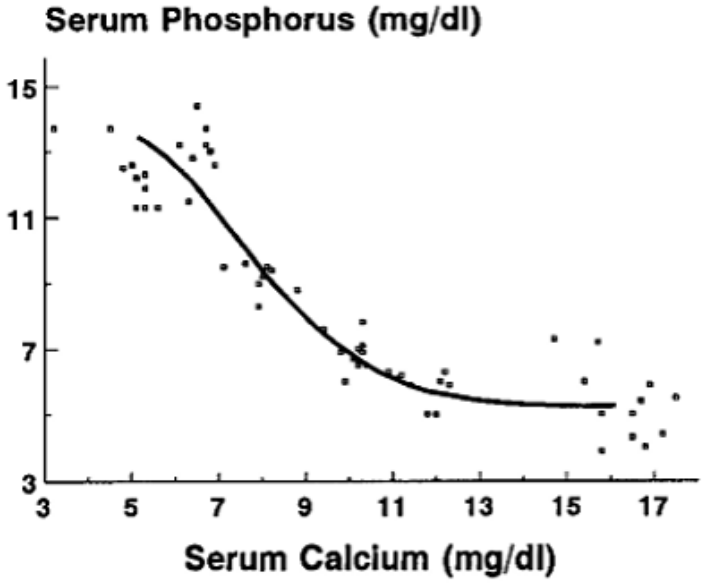
Our results from MR analysis support a causal role of serum P levels in coronary artery calcification in the general population, meaning that our findings were not driven by hyperphosphatemia, CKD or prevalent CVD. The sex-stratified MR analysis strongly suggests a more consistent causal association 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.

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9

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.

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Beyond boundaries: to the Priests - Mas allá de las fronteras: a los Sacerdotes
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"**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.
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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.