Sex- and Age-related Challenges in Calcium and Phosphate Homeostasis

Gender- en leeftijd- gerelateerde uitdagingen
in calcium en fosfaat homeostase

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Chapter 1

Introduction

Introduction

1. Bone metabolism

From its development and mineralization / ossification during gestation towards the end of life multiple processes take place in bone, making it a dynamic tissue [1]. In the embryonic stage bone develops in roughly two different ways, either via differentiation of the mesenchymal stem cell into osteoblasts, which are cells that are able to produce bone tissue (intramembranous bone formation), or via the ossification of cartilage (endochondral bone formation) after the mesenchymal stem cell has differentiated into a chrondrocyte [2-5]. After birth, bones grow longitudinally, gain mass, and are shaped through the process of modeling. Modeling is the formation of lamellar bone on bone surfaces by osteoblasts, and the removal of bone at other surfaces by osteoclasts, whereby osteoclast activity is independent from osteoblast activity [6]. When longitudinal bone growth ceases at the end of puberty, until around the age of 30 when peak bone mass is attained, the skeleton will continue to build up in both mass and strength. The maximum strength of the bone mass reached by an individual depends on genetics and environmental factors such as exercise and diet [7-12]. While bone formation and bone resorption are balanced until the 4-6th decade of life, a steady decline of bone mass occurs thereafter, with a more rapid decline in women around the time of menopause due to a sharp decrease in estrogen levels [13, 14].

1.1 Bone remodeling

Even after the attainment of peak bone mass bone remodeling (Figure 1) takes place, a process whereby osteoclasts remove damaged and brittle parts of bone, followed by osteoblast-mediated formation of new bone [15]. This process continues throughout life and is influenced and mediated by different cytokines, chemokines, and hormones [1, 11, 15, 16].

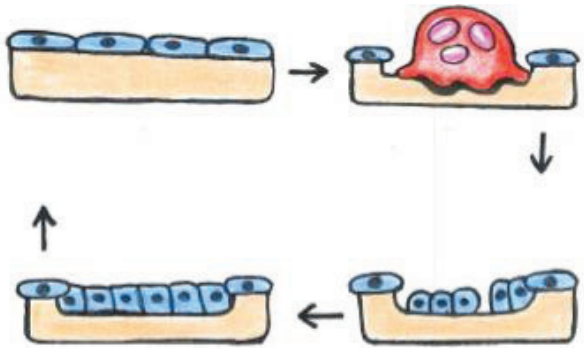

Figure1: schematic representation of bone remodeling

1.2 Osteoclasts, Osteoblasts and Osteocytes

There are three major cell types in bone: osteoblasts, osteoclasts and osteocytes. Osteoblasts are bone-forming cells. They are derived from mesenchymal stem cells and differentiate into osteoblasts via a complex mechanism involving several transcription factors such as runt-related transcription factor 2 (RUNX2), osterix (SP7), ß-catenin, low-density lipoprotein receptor-related protein 5 (LRP5) and the Wnt signaling pathway [17, 18]. Mesenchymal stem cells differentiate into osteoprogenitor cells, which will proliferate and differentiate into pre-osteoblasts able to produce alkaline phosphatase, an important enzyme for the generation of inorganic phosphate at this stage [19]. In addition, pre-osteoblasts start generating an extracellular matrix (ECM), which mainly consists of collagens such as collagen type I, as well as non-collagenous proteins. Upon differentiation of pre-osteoblasts into osteoblasts, the ECM is subsequently mineralized through formation of calcium and phosphate containing crystals (hydroxyapatite; $Ca_{10}(PO_4)_6(OH)_2$). The majority of the osteoblasts will undergo apoptosis or become bone-lining cells, while only a minority will be incorporated into bone as osteocytes [20]. Osteocytes are the most abundant cell types in bone. They are derived from the mesenchymal stem cell line and constitute terminally differentiated osteoblasts. To become embedded as an osteocyte in bone, an active invasive process cleaving collagens and other matrix proteins takes place, whereby the osteoblast transforms from a polygonal cell into a dendritic osteocyte [21]. Osteocytes are key signal transducers of mechanical loading. Mechanosensing results in the inhibition of the expression of sclerostin, an inhibitor of the Wnt-canonical signaling pathway, thereby leading to stimulation of osteoblast activity [22]. It is thought that osteocytes are the key regulators for bone remodeling due to the fact that they stimulate osteoclast formation and activation, as well as osteoblast and mesenchymal stem cell differentiation [23-25]. The discovery of osteocyte-secreted fibroblast growth factor 23 (FGF23) as a factor that plays an important role in proper calcium and phosphate homeostasis has renewed some interest in studying the metabolic roles of osteocytes in bone remodeling [22, 26, 27].

Bone resorption is primarily the function of osteoclasts. Osteoclasts are specialized multinucleated cells that are derived from the hematopoietic lineage. Their precursors are mononuclear cells, predominantly monocytes, that differentiate into osteoclasts in a paracrine manner by osteoblast-produced cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor-activator of nuclear factor kappa-B ligand (RANKL) [28-31]. Osteopro-

tegerin (OPG), which is also released by osteoblasts, serves as a decoy for the RANKL receptor, thereby blocking osteoclast formation and thus acting as a negative regulator of osteoclastic bone resorption [30, 31]. After fusion of the mononuclear precursors into multinucleated cells, osteoclasts are capable of attaching tightly to the bone surface done with a so-called sealing zone and resorb bone [28]. They do so by releasing H^+ and Cl^- ions into a resorption pit which is a lacuna underneath the osteoclast adjacent to the bone. These ions form acidic HCl and dissolve the inorganic bone matrix into calcium and phosphate molecules that are taken up by the osteoclasts and are excreted into the bloodstream [31]. In order to degrade the organic matrix, including collagens, osteoclasts release the enzyme cathepsin K, amongst others, into the resorption pit [32]. The initiation for development of osteoclasts is triggered by multiple hormones, of which parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are the most familiar ones [31]. It is possible to culture osteoclasts from human peripheral blood mononuclear cells [33]. Previously there have been two small studies evaluating differences in the ability of mononuclear cells to become osteoclasts between osteoporotic subjects and healthy controls [34, 35], D 4Amelio *et al*. found that both osteoclastogenesis and osteoclastic bone resorption was enhanced in osteoporotic women versus a control group [34]. Jevon *et al*. found no increase in osteoclastogenesis but increased osteoclastic bone resorption capacity in osteoporotic subjects versus healthy controls [35]. However, it is not known whether these differences in osteoclast formation ability and resorption capacity are still present in the case of longstanding osteoporosis when compared to healthy controls.

2. Osteoporosis

Osteoporosis is a condition characterized by low bone mineral density (BMD) and a deterioration of the bone microarchitecture, leading to frailty of bone and an increased risk of fractures [36]. Women are affected more often than men. This is partly due to the attainment of a lower peak bone mass in women. Furthermore, women have a rapid decline of estrogen levels after the menopause, resulting in increased bone resorption, which is not fully matched by bone formation. In 2015, the prevalence of osteoporosis in the Netherlands was 43.1 per 1000 for women and 7.5 per 1000 for men [37]. Osteoporosis is correlated with increased morbidity and mortality and a lower quality of life [38-40]. In the Netherlands in 2010, the direct costs dedicated to the treatment of osteoporosis were estimated at 200 million Euro [41].

In order to evaluate people at risk for fractures, BMD is measured at both the hip and the lumbar spine. Lower BMD is associated with an increased fracture risk. A BMD, (measured either at the femoral neck, lumbar spine or forearm) below -2.5 standard deviations (SD) of that of a 30-year-old with the same gender and race (T-score) is defined as osteoporosis by the World Health Organization (WHO) [42-44]. Moreover, the presence of a previous low-impact fracture of the spine, hip, wrist or humerus is a good predictor of consecutive fractures [45]. Therapy to reduce fracture risk is indicated when the T-score is below -2.5, when a T-score is below -1 in the presence of a vertebral fracture, or when there are additional risk factors like a recent fracture, disease and/or the use of medication associated with bone loss, recurrent falls and a T-score < -2, (CBO "richtlijn osteoporose en fractuur preventie 2011"). In order to identify those in need of therapy an online tool to calculate fracture risk (http://www.shef.ac.uk/FRAX/) was launched [46, 47]. Currently, the Dutch osteoporosis and fracture prevention guideline from 2011 advises the use of the FRAXporosis tool to calculate fracture risk current fallsst or um>91, without additional risk factors, to assess whether therapy should be indicated ("CBO richtlijn osteoporose en fractuur preventie 2011"). However, there are currently no cut-off levels defined in the Netherlands when using FRAX to commence treatment.

3. Calcium and phosphate metabolism and its regulating hormones

Calcium and phosphate are stored in bone in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which constitutes the vast majority of both ions in the body [48]. Calcium and phosphate are vital for many processes in the body. Calcium plays an important role in blood coagulation, nerve excitability, muscle contraction, membrane permeability and stability, secretion of various hormones, and various other processes related to the aforementioned. Calcium levels in the circulation are tightly regulated [49]. In order to maintain stable serum calcium concentrations the parathyroid glands contain the calcium-sensing receptor (CaSR). The CaSR senses the free concentration of calcium in the blood and subsequently responds by either increasing or decreasing the secretion of PTH from the parathyroid glands [50-52]. When calcium levels decrease, increased PTH levels leads to both increased calcium re-absorption in the kidney and to increased bone resorption, thereby releasing calcium and phosphate from the bone into the bloodstream. Furthermore, PTH induces the 1α-hydroxylase enzyme in the kidney, thereby enhancing the conversion of 25-hydroxyvitamin D_3 into its active form

1,25-dihydroxyvitamin D_3, which results in increased intestinal calcium (and phosphate) absorption [53].

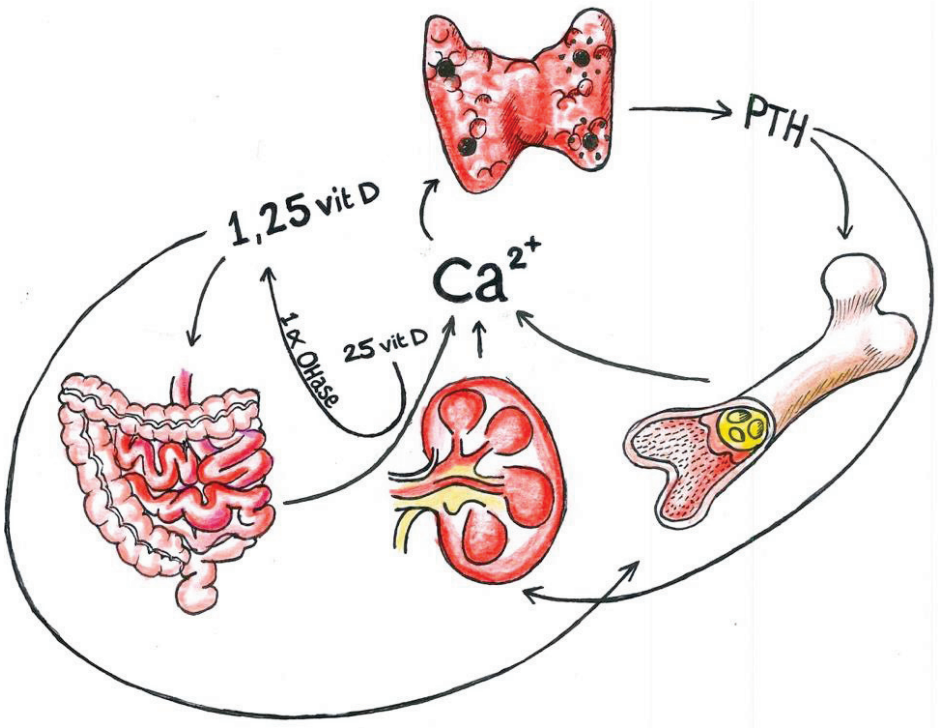

Figure 2: schematic representation of hormones and organs involved in the maintenance of adequate serum calcium levels. In response to decreased free calcium concentrations PTH is secreted from the parathyroid glands. PTH stimulates calcium release from the bone and it stimulates calcium reabsorption in the kidney. Furthermore, PTH induces the 1α-hydroxylase enzyme. The 1α-hydroxylase enzyme stimulates the hydroxylation of 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3, which has the ability to increases intestinal calcium absorption.

Phosphate is an important component of the backbone structure of DNA and several other types of molecules such as phospholipids. Moreover, it is an important molecule in energy consuming processes in the body by being part of adenosine triphosphate (ATP). Despite phosphate being less tightly regulated compared to calcium, PTH and 1,25-dihydroxyvitamin D_3 are also essential in the maintenance of stable serum phosphate concentrations [54]. On the one hand, PTH and 1,25-dihydroxyvitamin D_3 stimulate phosphate release from the bone and intestinal phosphate absorption; on the other hand PTH facilitates phosphate excretion via the kidneys [53-55]. Recently, FGF23 was shown to play a crucial role in phosphate metabolism as well. It is predominantly produced by osteoblasts and osteocytes, and inhibits renal tubular phosphate reabsorption and PTH synthesis. Furthermore, it inhibits the

conversion of 25-hydroxyvitamin D_3 into its active form 1,25-dihydroxy-vitamin D_3 by reducing 1α-hydroxylase synthesis [53].
With aging, alterations occur in calcium, phosphate, and bone homeostasis [56-59]. Both sexual dimorphism in phosphate levels and bone homeostasis with aging (e.g. menopause) have been extensively studied [59-64], but there have been contradicting and less comprehensive data on sexual dimorphism of calcium homeostasis [60, 65-69]. Additionally, little is known how the sex hormones estradiol and testosterone influence calcium and phosphate metabolism in men and women at older age.

Phosphate levels and health
Phosphate is present in numerous food products, especially in protein rich food products such as meat and dairy, as organic esters and only about 40 to 60 percent of this form of phosphate are absorbed in the intestines [70]. Phosphate is also supplemented as phosphoric acid, phosphates and polyphosphates to a large number of food products ranging from soft drinks and meat products to pre-packed food items [71]. These non-organically bound forms of phosphate are easily absorbed in the intestines and multiple reports have indicated that this absorption can increase serum phosphate levels [72]. High phosphate levels can lead to ectopic calcifications e.g., in arteries [73]. Several studies have reported increased cardiovascular morbidity and mortality related to higher phosphate levels in patients with chronic kidney disease [74, 75], and recently even in persons without chronic kidney disease [62, 64, 76]. Based on these findings, Ritz *et al.* raised awareness about the possible detrimental effects of phosphate additives in food for the general population [72]. The European Food Safety Authority (EFSA) set out to evaluate the concerns postulated by Ritz *et al.* and found only little evidence for detrimental health effects of phosphate additives, disallowing advise regarding the restriction of the usage of the aforementioned phosphate additives at this point in time. However, they will keep a close watch and have said to re-evaluate phosphates in food additives in 2018, as they will have gathered more data by then [71]. To date, it is unclear whether a relationship exists between phosphate levels, with either BMD or fracture risk at population level.

4. Vitamin D

Vitamin D is a steroid hormone that is synthesized in the body by a series of metabolic reactions, which start in the skin under the influence of sunlight, and it can be derived from nutritional sources and supplements. One of the

first metabolites in the body for the formation of Vitamin D is 7-dehydrocholesterol, even though the mechanism and location of its synthesis are still a matter of debate [77]. Under the influence of UV-B light 7-dehydrocholesterol is converted into pre-Vitamin D_3, which under influence of heat rapidly transforms into Vitamin D_3 (cholecalciferol). Vitamin D_3 is bound to vitamin D binding protein in the circulation and can be stored in body fat. Next, 25-hydroxyvitamin D_3 is formed by hydroxylation of cholecalciferol in the liver [78] and constitutes the major circulating form of Vitamin D. Upon demand of the body, 25-hydroxyvitamin D_3 is further hydroxylated to 1,25-dihydroxyvitamin D_3 through 1α –hydroxylase (CYP27B1), an enzyme most predominantly expressed by the kidney but also reported in multiple other cell types throughout the body [79, 80]. Being the most bioactive form of vitamin D, 1,25-dihydroxyvitamin D_3 signals in target cells by binding to the vitamin D receptor (VDR), a member of the nuclear receptor family. After binding to the VDR, VDR heterodimerizes with retinoid X receptor (RXR) and subsequently binds to vitamin D responsive Elements (VDREs) of target gene promoters in order to regulate their transcription. These include FGF23 and RANKL, which play a role in the regulation of calcium and phosphate homeostasis [81]. Besides, 1,25-dihydroxyvitamin D_3 controls both its own level and activity in all its target cells by stimulating 24-hydroxylase (CYP24A1), which initiates the conversion of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 [78] into less active forms of vitamin D.

Whilst 1 α-hydroxylase in the kidney is responsible for the majority of the circulating 1,25-dihydroxyvitamin D_3, the presence of 1α-hydroxylase in other cell types and tissues is thought to have a more auto- or paracrine function [79, 80].

During aging, vitamin D deficiency may occur due to diminished synthesis in the skin as a result of decreased exposure to sunlight, which can be observed in nursing home populations, but also the decreased ability of the skin to synthesize vitamin D [82]. The bioavailability of vitamin D can lessen with age as the result of decreased intestinal uptake due to intestinal microvilli atrophy, and a reduced synthesis of 1,25-dihydroxyvitamin D_3 due to decreased kidney function might also be observed with senectitude [78, 83, 84]. The combination of the factors mentioned above predisposes elderly subjects to vitamin D deficiency, resulting in increased risk of osteoporosis and disturbances in calcium- and phosphate-metabolism.

5. TRPV5 and aging

Transient Receptor Potential channel V5 (TRPV5) is a member of the TRP superfamily, which is a group of highly homologous genes involved in the active transport of ions across the cell membrane. The *TRPV5* gene encodes a calcium-selective channel present at the luminal site in the distal convoluted tubule and the connecting tubule cells in the kidney [85], where it is responsible for calcium reabsorption from urine. Besides, it is located at the ruffled border membrane in osteoclasts, where bone resorption takes place, and may well be involved in calcium transport from the bone towards the circulation. Hence, mice lacking TRPV5 display hypercalciuria with compensatory increased intestinal calcium absorption through vitamin D-induced upregulation of the TRPV5 homologue TRPV6. In addition, these mice display a bone phenotype of reduced trabecular and cortical bone thickness [86-88], suggesting that TRPV5 deficiency does not only affect calcium homeostasis, but might also have a direct effect on bone homeostasis. Although the function of TRPV5 is known, it is currently unclear what the impact of TRPV5 deficiency is on calcium homeostasis and bone metabolism within the process of aging.

6. Aims and scopes of this thesis

Our understanding of the variations in calcium and phosphate homeostasis throughout the lifespan is currently incomplete. As alluded to before, bone health is vital for healthy aging, and calcium and phosphate are crucial components that are incorporated in bone as hydroxyapatite. Disturbances in their regulation are associated with aging-related diseases. The general aim of this thesis was to study calcium and phosphate homeostasis in relation to aging and to the age-related disorder osteoporosis. Firstly, we addressed sexual dimorphisms of calcium and phosphate homeostasis by comparing calcium and phosphate homeostasis between men and women (**Chapter 2**) above 45 years of age. For this we used three population-based cohorts of community dwelling subjects from the Rotterdam Study, with ages ranging from 45 to 99 years [89]. In **Chapter 3** we addressed alteration in serum calcium and phosphate levels over time by assessing three cohorts derived from hospital records of Erasmus MC in 2005, 2010 and 2014, with ages ranging from 1 until 97 years of age. In **Chapter 4** we assessed whether differences in phosphate levels influenced BMD and fracture risk. In order to do so, we studied serum phosphate levels in relation to BMD and fractures in three different cohorts from the Rotterdam study [89] and one cohort from the Osteoporotic Fractures in Men (MrOs) study [90, 91]. The three different

cohorts from the Rotterdam Study are the same cohorts as described in Chapter 2. The Osteoporotic Fractures in Men (MrOs) study is a population based study with the aim to identify those factors influencing fracture risk in 5994 male subjects aged 65 years or older [90, 91]. We were able to include sex-specific effects on the relation of phosphate levels with BMD and fracture risk exclusively in the Rotterdam study. In **Chapter 5** we described the effects of genetically defined lactose intolerance on calcium and bone metabolism, as it can influence calcium intake, [92, 93]. This was performed by associating the T-13910C polymorphism upstream of the Lactase Phlorizin Hydrolase gene (*LPH)* with bone parameters, including fracture risk, BMD, and bone size and geometry, as well as calcium and vitamin D metabolism. Moreover, we assessed gene interaction between the T-13910C *LPH* polymorphism and genetic variations in the *VDR* gene to assess whether minor but lifelong differences in the handling of calcium and vitamin D can affect bone parameters. We made use of the same elderly population from the Rotterdam Study as described in Chapter 2 and a different elderly population cohort from the Longitudinal Aging Study Amsterdam (LASA). In **Chapter 6,** we assessed a patient with severe vitamin D deficient rickets due to a mutation in the 1α -hydroxylase gene *CYP27B1*, and investigated the ability of peripheral mononuclear cells (PBMCs) to convert 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3. We also compared the ability of both parents, who were carrier of only one mutation, and healthy controls for their ability to convert 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3.

In **Chapter 7,** studies on PBMC-derived osteoclast formation and osteoclast activity *in vitro* in two extreme bone phenotypes are described. We studied women, on average 25 years after menopause, with osteoporosis with at least 1 fracture, and compared their osteoclast formation and osteoclast activity *in vitro* with age-matched healthy controls. In **Chapter 8,** we described a study on a TRPV5 deficient mouse model with disturbed calcium homeostasis. We assessed the impact of TRPV5 deficiency on calcium homeostasis and bone metabolism during aging. Amongst other analyses, we focused on bone microstructure and mineralization, as both are potentially affected as a consequence of disturbed life-long calcium challenges. **Chapter 9** contains a general discussion and presents future perspectives. **Chapter 10** concludes with a summary and presents the conclusions of this thesis. **Chapter 11** presents the summary and conclusions in Dutch.

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Chapter 4

Serum phosphate is associated with fracture risk: The Rotterdam Study and MrOS

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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 meta-analyses. 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 (ß: -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 rick-

ets/osteomalacia [5, 6]. On the other hand, extreme hyperphosphatemia induces tumoral calcinosis, characterized by ectopic calcifications but also mineralization defects [7-9].

The recent finding that P regulation is exerted also by the phosphatonins α-Klotho and the osteocyte-derived fibroblast growth factor 23 (FGF23) has established the concept that bone is not only a P reservoir but also acts as an endocrine organ, regulating P levels and mineralization [3, 7, 10]. Therefore, a potential bidirectional relationship between P levels and bone can be postulated, in which adequate P availability allows bone mineralization while osteocytes regulate P levels through FGF23 synthesis and through master control of bone remodeling [1, 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 (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 or more) and in the US Osteoporotic Fractures in Men (MrOS) study (recruitment period 2000-2002, original n = 5994; all male subjects aged 65 or older) [17-19]. 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 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, ßC-terminal cross-linked telopeptide of type I collagen (ßCTX, Roche Diagnostics) was measured, with intra- and interassays CVs < 4.2% [24].

DXA scanning

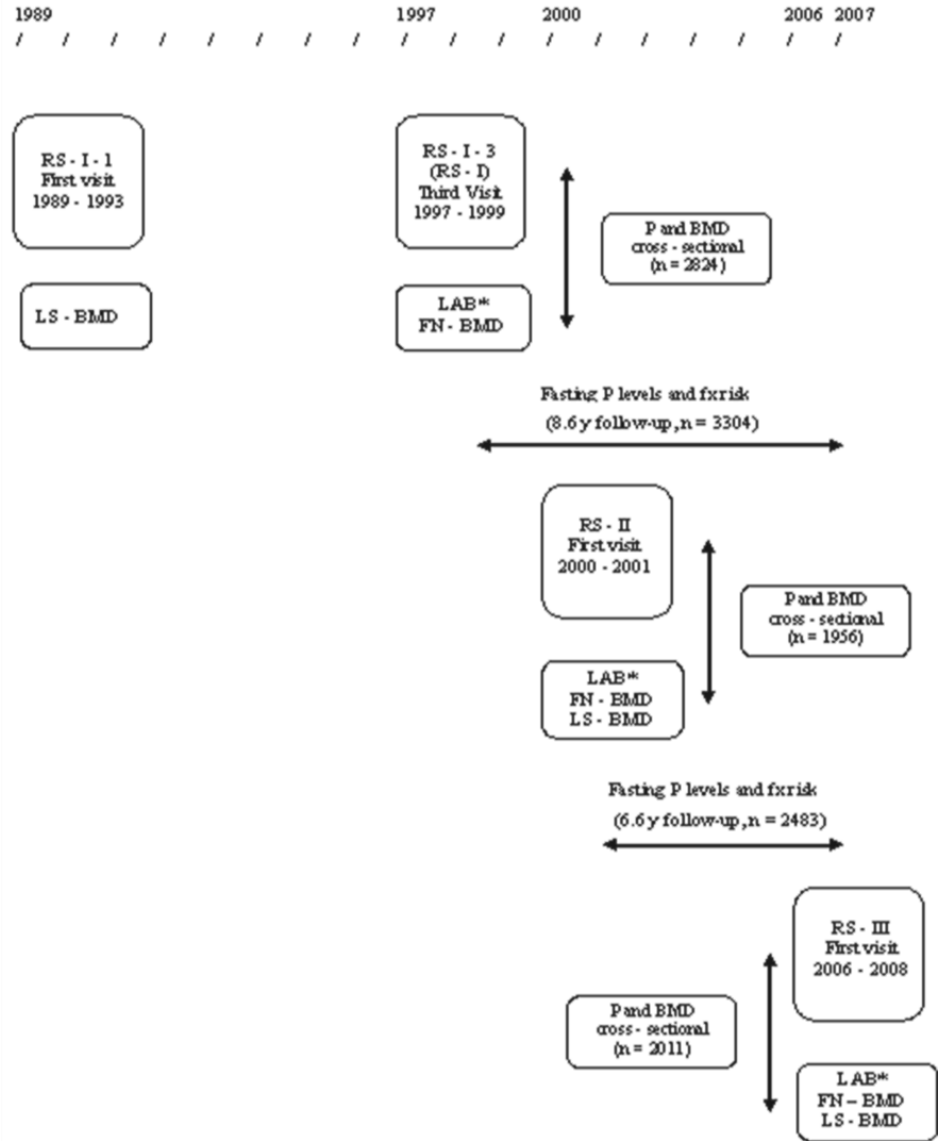
Trained radiographic technicians performed BMD measurements using dual-energy X-ray absorptiometry (DXA). RS-I participants were assessed at baseline (lumbar spine-LS-BMD, RS-I-1, 1989-1991) and at the third visit (femoral neck FN-BMD, RS-I, 1997-1999), whereas RS-II and RS-III participants were assessed 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 in RS-III (GE Lunar Corp, Madison, WI, USA) [25]. 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, 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 [26, 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.

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

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 dataset. 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 we compared its distribution across sexes in the Rotterdam Study applying *t* tests [33]. 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 and the Modification of Diet in Renal Disease (MDRD) study equation were applied to estimate eGFR (mL/min) in the Rotterdam Study and MrOS, respectively [35, 36]. 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 (1mg/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 (ß) are expressed per 1 mg/dL increase in P levels. Fitness of different models was compared through the Akaike Information Criteria AIC; linear models with normal distributions displayed lower AIC values, corresponding to a better fit [37]. 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; site and race were also included in MrOS (Model I) [40-42]. 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 disease 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 and in the association of P with several outcomes we explored relations of P with bone traits in sex-combined and in sex-stratified models in RS cohorts [15, 16, 33, 43].

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).

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**
I) RS-I												
N Mean (mg/dL)	242 (2.56)	243 (2.92)	243 (3.14)	243 (3.37)	243 (3.76)		322 (3.02)	322 (3.40)	322 (3.62)	322 (3.84)	322 (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 (y)	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
II) RS-II												
N Mean (mg/dL)	181 (2.48)	182 (2.84)	182 (3.06)	182 (3.29)	182 (3.70)		209 (2.91)	209 (3.31)	210 (3.52)	209 (3.76)	210 (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 (y)	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
III) RS-III												
N Mean (mg/dL)	174 (2.56)	174 (2.94)	174 (3.20)	174 (3.45)	174 (3.87)		228 (2.97)	228 (3.39)	228 (3.63)	228 (3.85)	229 (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 (y)	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/cm^2)	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
Creatinine (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+ (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

* *P* values corresponds to age-adjusted significance of trend across quintiles. BMI: body mass index. Smoke: ever smoke. 25OHD: 25-hydroxyvitamin D levels; FN-BMD: femoral neck bone mineral density; prevalent DM: prevalent diabetes mellitus; creatin: creatinine; 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

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 ß [95% CI]) (ß: -0.04 [-0.08 to 0.01], $p = 0.096$). In women, a negative association was found in the age-only adjusted model (ß: -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 (ß: -0.06 [-0.11 to -0.02], $p = 0.007$), which was driven by men from RS cohorts (ß: -0.12 [-0.19 to -0.04], $p = 0.002$) and not significant in men from MrOS (ß: -0.03 [-0.09 to 0.03], $p = 0.360$). In women, a negative association was found in the age-adjusted model (pooled ß: -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 (ß: -0.06 [-0.09 to -0.02], $p=0.004$) was driven by a significant negative association in men.

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						
N Mean (mg/dL)	1086 (2.6)	1085 (2.9)	1085 (3.2)	1085 (3.4)	1084 (3.8)	
Range	1.8-2.8	2.8-3.0	3.1-3.3	3.3-3.5	3.5-6.8	
Age (y)	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

**P* values corresponds to age-adjusted significance of trend across quintiles
eGFR: estimated glomerular filtration rate according to Modification of Diet in Renal Disease (MDRD) study equation

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 ($p_{\text{heterogeneity}} = 0.258$).

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

	Model I			Model II		
	n	*ß* (95% CI)[a]	*p*	N	*ß* (95% CI)[a]	*p*
RS-I						
Men	1214	-0.11 (-0.24 to 0.01)	0.084	1204	-0.06 (-0.18 to 0.06)	0.328
Women	1610	-0.24 (-0.35 to -0.13)	<0.001	1596	-0.05 (-0.16 to 0.05)	0.314
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	12216	-0.08 (-0.11 to -0.04)	<0.001	12177	-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
Studiescombined[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	12625	-0.10 (-0.13 to -0.06)	<0.001	12528	-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[b]						
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/ 11196	1.45 (1.31-1.62)	<0.001	1608/ 9716	1.47 (1.31-1.65)	<0.001

n_o fxs: number of fractures

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 combined applying a fixed effects model

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 [1.08-2.09], 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 trend < 0.001).

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 / n_o risk**	**HR**[b,c] (95% CI)	***p***	**P levels**[a] mean (range)	**events / n_o 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 associa-

tions were found for (clinical) vertebral fractures in men while women displayed a stronger association for humerus fractures.

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/ n_o 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

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 ≤ 58mL/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 HR = 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.

The same pattern was observed after stratification by kidney function (eGFR 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 ßCTX 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 ßCTX 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 re-

mained 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 compared with subjects without fractures, but to the best of our knowlegde, no prospective studies have been reported at the population level [13, 14, 45].

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, through P-dependent and independent effects [6, 7, 46-48]. However, consistent with previous research, adjustments for FGF23 levels did not influence the association of P with fracture risk in men from MrOS [49].

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, even without overt hyperphosphatemia [51]. This finding was not abol-

ished 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 and that it tends to cluster in osteocytes near the trabecular periphery and the lacuna-canalicular systems, in contrast to the expression pattern of other osteocytes (DMP1
osteocytes), which are diffusely located throughout bone [53-58]. This observation needs to be tested in other cohorts and if confirmed, it deserves further research. Because LS-BMD measurements can be affected by degenerative changes, 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, 59, 60].

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 which may not be reflected as fasting P [66]. 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 and it may have important clinical consequences for subjects with and without CKD [13, 14].

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.

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Supplemental Tables

Supplemental Table S1. 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-combine	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)

Supplemental Table S2. 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		
	$n_o$fxs /total n	**HR**[1,2] (95% CI)	***p***	**$n_o$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
Studiescombined						
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

Supplemental Table S3. 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

Supplemental Table S4**.** 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

Chapter 5

The T-13910C polymorphism in the lactase phlorizin hydrolase gene is associated with differences in serum calcium levels and calcium intake

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Abstract

The C-variant of a T-13910C polymorphism (rs4988235; NT_022135. 15:g.25316568G > A) upstream of the lactase phlorizin hydrolase (LPH) gene causes lactose intolerance. Association studies with differences in bone parameters and fracture risk have been inconclusive. The objective of this study was to examine the association of LPH rs4988235 with body height and bone parameters and calcium homeostasis in two elderly populations of Dutch Caucasians and assess interaction with vitamin D receptor (VDR) polymorphisms.

Genotyping of LPH and VDR polymorphisms was performed in 6367 individuals from the Rotterdam Study and 844 from the Longitudinal Aging Study Amsterdam (LASA). Associations with age, height, weight, bone mineral density (BMD), skeletal morphometric parameters and serum vitamin D and calcium levels, and dietary calcium intake were assessed using ANOVA or analysis of covariance, and allele dose effect was assessed using linear regression analysis. Fracture risk was analyzed using Cox's proportional hazard regression analysis. Associations with body height ($p = 2.7x10^{-8}$) and vertebral area ($p = .048$) found in the Rotterdam study were explained by population stratification, as assessed by principal component analyses, and disappeared after additional adjustments. No associations with femoral neck or lumbar spine BMD or with fracture risk were detected. Calcium intake and ionized serum calcium were significantly lower in C-homozygotes ($p = 9.2x10^{-7}$, $p = .02$, respectively). For none of the parameters studied was interaction between the T-13910C polymorphism and VDR block 5 haplotype 1 observed. We show that the C-allele of the T-13910C polymorphism causing lactose intolerance is associated with lower dietary calcium intake and serum calcium levels but not with BMD or fractures. The associations observed with height and vertebral area were the result of population stratification. This demonstrates the impact of population stratification and urges researchers to carefully take this into account in genetic associations, in particular, in dietary intake-related phenotypes, of which LPH and lactose intolerance are a strong example.

Introduction

Lactose intolerance (OMIM 603202) is a condition in which lactose is not converted into galactose and glucose owing to decreased intestinal lactase levels. Consequently, the undigested lactose causes gastrointestinal com-

plaints that lead to avoiding consumption of milk and milk products and therefore to lower calcium intake [1-3].

Lactose intolerance evolves in infancy after weaning when lactase enzyme activity is downregulated. In 2002, Enattah and colleagues identified a polymorphism, NT_022135.15:g.25316568G > A, 13910 bp upstream of the lactase phlorizin hydrolase (LPH) gene (2q21) that is responsible for lower lactase enzyme activity [4]. This polymorphism will be referred to as T-13910C throughout the article. C-homozygotes are 100% associated with lactase nonpersistence, that is, genetically defined lactose intolerance. The current thought is that the T-variant became established in response to changing dietary habits during the early days of cattle, sheep and goat herding when populations started to consume milk containing lactose as part of their dietary habits [5]. The prevalence of lactose intolerance worldwide shows a distribution pattern that is associated with dietary intake habits [5, 6]. In Europe, this is represented by a rather steep incline in the prevalence of lactose intolerance from northern toward southern Europe, with a prevalence ranging from 2% in Sweden up to 70% in Italy and Turkey [5].

Osteoporosis is a multifactorial disorder caused by the interaction of genetic and environmental factors such as body weight and nutrition. Important nutrients for bone metabolism are calcium and vitamin D. Because of the importance of calcium intake for bone metabolism, one can hypothesize that the C-allele is associated with decreased bone mineral density (BMD) and, as a result, also with increased fracture risk. Yet no consistent results in epidemiological studies have been found. Table 1 summarizes the outcome of several studies that assessed lactose intolerance with BMD and / or fracture risk in countries with high (Finland) or low (Austria) calcium intake [7-11].

The vitamin D receptor (VDR) gene mediates the actions of the vitamin D endocrine system in calcium homeostasis and bone metabolism [12-14]. VDR knockout mice suffer from hypocalcaemia, hyperparathyroidism, rickets, and osteomalacia, a phenotype that can be rescued when these mice are fed a high-calcium, high-lactose, and high-phosphorus diet [15, 16]. In a previous examination of VDR polymorphisms in relation to osteoporosis in the Rotterdam Study, VDR block 5 haplotype 1 was associated with increased fracture risk [17]. In a functional study, the VDR block 5 haplotype 1 allele was found to be associated with a 15% lower VDR mRNA expression level than block 5 haplotype 2 [17]. Therefore, we hypothesized that carrying VDR block 5 haplotype allele 1 in lactose-intolerant subjects, that is, LPH C-homozygotes

subjects, could result in even stronger associations with BMD and / or fracture risk.

In this study we have evaluated the influence of genetically defined lactose intolerance on bone parameters, including fracture risk, BMD, and bone size and geometry, and on calcium and vitamin D metabolism in both genders in a large prospective population-based study of elderly Dutch Caucasians with relatively high dietary calcium intake. Furthermore, we assessed gene interaction between the T-13910C LPH polymorphism and genetic variations in the VDR gene.

Table 1: Overview of studies where lactose intolerance was assessed in relation to bone parameters

Study	Sample size (n)	Mean age (age range)	Gender	Diagnostic tool of Lactose intolerance	Main outcome
Ennattah et al., 2005 [31] (Finland)	483	89 (85-98)	♂ 106 ♀ 377	Allelic discrimination	Increased risk for hip and wrist fractures in C-homozygotes. No difference in self-reported milk consumption between the genotypes.
Obermayer-Pietsch et al., 2004 [11] (Austria)	258	62 ± 9	♀ 258	Allelic discrimination	Increased fracture risk and lower BMD for C-homozygotes. 55% lower calcium intake from milk but not from yoghurt and other nutrients
Gugatschka et al., 2005 [8] (Austria)	228	56 ± 12	♂ 278	Allelic discrimination	No associations with BMD parameters and anthropometric characteristics found. No differences in overall calcium intake, calcium intake from milk and calcium intake from yoghurt.
Kudlacek et al., 2002 [34] (Austria)	218	58.2 ± 11.5	♂ 43 ♀ 175	H_2 breath hydrogen test	No difference in bone loss. Calcium intake from milk lower in lactose malabsorbers.
Honkanen et al., 1996 [9] (Finland)	2025	53.5 ± 3.0 (48-95)	♀2025	Self-reported lactose intolerance	Lower BMD at the site of the femoral neck and at the lumbar spine.

Material and Methods

Rotterdam Study population

Subjects were participants of the Rotterdam Study, a large prospective population-based cohort study of white subjects aged 55 years and older living in the Ommoord district of Rotterdam, The Netherlands [18]. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus University Medical Center, and written informed consent was obtained from each subject. This study is based on 6146 subjects (3777 females) for whom genotype data were available for the LPH T-13910C polymorphism.

Clinical examination

Height and weight were measured in the standing position wearing indoor clothing without shoes. Body Mass Index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2)

At baseline, BMD (expressed in g/cm^2) was measured at the site of the femoral neck and lumbar spine. The average vertebral area (cm^2) was measured over the L2-L4 region of the lumbar spine by posteroanterior scanning using dual energy X-ray absorptiometry (DEXA, Lunar DPX-L Densitometry, Lunar Corp., Madison, WI, USA), as described previously [19]. Hip structural analysis also was assessed as described previously [20].

Vertebral fracture assessment and incident nonvertebral fracture assessment

The assessment of vertebral fractures and incident nonvertebral fractures was described in detail previously by Rivadeneira and colleagues. [20].

Dietary assessment of semiquantitative food frequency questionnaire (SFFQ)

Nutrient intake assessment in the Rotterdam Study was performed in two steps. First, a modification of a mailed self-administered semiquantitative food frequency questionnaire was sent to subjects. Second, a dietary assessment was done by a dietician during a visit of the subject to the research facility that took place about 22 days after the first step [21]. Sources of calcium intake were divided into three types of food groups: milk, milk derivatives and nonmilk derivatives. Calcium intake from milk constitutes "raw" milk, fat milk, half-fat milk and nonfat milk. Milk derivatives are modified milk products such as yogurt, buttermilk, sour cream, and so on. Products in the nonmilk-derivatives group include fruit, vegetables, nuts, and cheese, among others. This subdivision is based on the Dutch Food Composition As-

sociation (NEVO) tables of 1993 [22]. The conversion from foods to energy and nutrient intake was established with a computerized version of the Dutch Food Composition table [22].

Serum measurements

Serum calcium was measured with a colorimetric detection assay using the Hitachi 917 (Roche, Mannheim, Germany). ^{125}I radioimmunoassay (RIA) was used for the quantitative determination of serum 1α-25-dihydroxyvitamin D^3 (1,25(OH)$_2$-D$_3$; IDS, Boldon, UK) and serum 25-hydroxyvitamin D (25-(OH)D$_3$; DiaSorin, Stillwater, MN, U.S.A).

LASA population

The Longitudinal Aging Study Amsterdam (LASA) is an ongoing cohort study of elderly persons aged 55 to 85 years. The sampling and data-collection procedures have been described in detail elsewhere [23]. In brief, a random sample stratified by age, sex, and expected 5-year mortality rate was drawn from the population registers of 11 municipalities in three regions of the Netherlands. In total, 3107 persons were enrolled in the baseline examination in 1992-1993. For this study, persons who participated in the medical interviews were selected. Of these, 844 subjects were genotyped for the LPH T-13910C polymorphism. The study was approved by the Medical Ethics Committee and all participants signed informed consent

Fracture assessment.

Information on incident fractures and falls was obtained for 1289 respondents between the second cycle of data collection in 1995-1996 and the third cycle of data collection in 1998-1999.

Serum measurements

Blood samples were obtained in 1995-1996. Serum 25-(OH)D$_3$ was measured according to a competitive binding-protein assay (Nichols Diagnostics, San Capistrano, CA, USA).

Genotyping

Genotyping of Rotterdam Study and LASA participants were performed in the same laboratory (Department Internal Medicine, Erasmus MC, Rotterdam). Details on genotyping procedures are described previously by Stolk and colleagues [24]. Assay-by-design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay (Applied Biosystems Inc., Foster City, CA, USA) for the LPH T-13910C polymorphism

(Forward primer: CTCTGCGCTGGCAATACAG; Reverse primer: AAATGCAACCTAAGGAGGAGAGTTC); FAM (carboxyfluorescein dye) probe: ATAAGATAATGTAGTCCCTGGC; VIC (a proprietary fluorescent dye) probe: ATAAGATAATGTAGCCCCTGGC.
VDR genotypes in the 3' UTR region were determined, and haplotype blocks were constructed as described in detail by Fang and colleagues [25].
To address the possibility of genotyping errors, 5% of the Rotterdam Study and the LASA was regenotyped for the LPH T-13910C polymorphism.

Data analysis

Subjects were classified according to LPH genotypes, with the TT and TC genotypes being lactose tolerant and the CC genotype being lactose intolerant [4].
We allowed for three possible models to explain differences between groups, that is, an allele dose effect, a dominant effect, or a recessive effect. Allele dose was defined as the number of copies of a certain allele in the genotype. In case of a dominant or recessive effect of the test allele, analysis of (co)variance [AN(C)OVA] was performed to test for differences between two genotype groups. To analyze dominant effects, we compared C-allele carriers versus non-C-allele carriers, while for recessive effects, homozygous subjects for the C-allele were compared with heterozygous carriers combined with noncarriers. All analyses were adjusted for age and gender**.**
Allele and genotype frequencies of the polymorphisms were tested for Hardy-Weinberg equilibrium proportions using the ARLEQUIN package [26].
Data of the Illumina array (Illumina Infinium HumanHap550 Chip Array [27]) containing 561466 single-nucleotide polymorphisms (SNPs) were used to study the presence of population stratification and unrecognized relationships between individuals. The possibility of population stratification affecting our results was assessed from the genomic inflation factor λ [28]. obtained from the genome-wide analysis of body height in the Rotterdam Study. In addition, considering that the relation of this LPH polymorphism with height is known to be subject to important stratification [29], we corrected the analyses using principal components (PC) to minimize the possibility of spurious findings owing to population-stratification effects. The PC method allows one to correct for ancestry differences leading to spurious associations in disease-association studies [30]. Four PCs derived from genome-wide information were estimated from multidimensional scaling of the identity-by-state (IBS) pair-wise distances between Rotterdam Study individuals. All subsequent analyses in the Rotterdam Study were adjusted for

these four PCs. To estimate nonvertebral fracture risk, we used Cox proportional-hazard models, thereby taking potential differences in follow-up into account. To estimate the risk of vertebral fractures, odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using logistic regression models. p Values were two-sided, and .05 or less was considered significant.

To assess differences in the effects of the LPH T-13910C polymorphism analyses among carriers and noncarriers of the block 5 haplotype 1 of the VDR gene, an interaction term was included together with main effects in the models.

All statistical analyses were performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Genotyping showed a 31% and 29% allele frequency of the T-13910C polymorphism in the Rotterdam Study and in LASA, respectively, with no significant differences between genders; prevalence of the C-homozygous genotype was about 10% in both populations (Table 2). The genotype distribution of the T-13910C polymorphism did not follow Hardy-Weinberg equilibrium proportions in the Rotterdam Study (p = .0001) and in LASA (p = .03) No genotyping errors were observed after regenotyping 5% of both populations. To address the possibility of population admixture affecting the results, the genomic inflation factor λ for height was calculated in the RotterdamSstudy.

Table 2: LPH T-13910C genotype frequencies in the Rotterdam Study and in the LASA population

	Genotype					
	TT	**TC**	**CC**	**T-allele**	**C-allele**	**HWE***
Rotterdam Study						
Men	1248(48%)	1061(41%)	281(11%)	69%	31%	P<0.0001
Women	1816(48%)	1577(42%)	384(10%)	69%	31%	
LASA						
Men	227(55%)	149(36%)	39(9%)	73%	27%	P<0.03
Women	208(48%)	175(41%)	46(11%)	69%	31%	

*HWE: Hardy-Weinberg equilibrium

The genomic inflation factor for body height was 1.013, which, being close to unity, is unlikely to reflect important population stratification. The genomic inflation factor for BMD was also close to unity, as reported by Richards and colleagues. [27]. Despite the low genomic inflation, as assessed by the λ, we

identified by PC analyses four independent components. The second and third PCs were shown to be significantly associated with differences in height ($p = 2.8x10^{-5}$ and $p = 3.4x10^{-23}$, respectively) but with none of the other endpoints examined. The other components (C1 and C4) were not associated with height or any of the other endpoints presented in this study. However, since all components are independent, we corrected all analyses for all four components.

LPH genotype and height

The baseline characteristics of both study populations are described in Table 3. In the Rotterdam Study, the T-13910C polymorphism was significantly associated with height, with CC-homozygotes having a lower height ($p = 4.8x10^{-8}$). The association was driven by principal components C2 and C3 and was lost after adjustment (p = .72), indicating that the initial association likely was driven by population-stratification effects (Table 3).

A significant association with weight (p = .0006) was found, with C-homozygotes having lower weight than carriers of the T-allele. Adjustment for the PCs (ie, height) showed this association to be due to stratification effects as well (p = .18). For BMI, no association was found for the T-13910C polymorphism in either men or women.

In the LASA, no associations with weight and BMI were found (Table 3). An allele dose effect for height towards a smaller stature for the C-allele was observed (1.7 cm, 1.1%, p trend = .048) but not for weight and BMI. In the LASA, we did not have the means to correct for possible stratification effects.

LPH genotype and bone-related end-points

No significant associations with femoral neck or lumbar spine BMD were detected in either the Rotterdam Study or the LASA (Table 3). When studying the femoral bone geometry data from the Rotterdam Study, that is, narrow neck width, cortical thickness, and the buckling ratio, only narrow neck width was significantly associated with LPH (p = .004) and remained significant after adjustments. The vertebral area also was associated with the LPH polymorphism (p = .048). Similar to height, the association with vertebral area was driven by the components C2 and C3 (p = .62) after adjustment for population stratification. No associations with vertebral or osteoporotic fractures were found in the Rotterdam Study or with osteoporotic fractures in LASA (data not shown).

Table 3: Baseline characteristics of the study populations by LPH T-13910C genotype

	Total	TT	TC	CC	ANOVA		
Rotterdam Study	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	p-value crude	p-value adjusted	p-value adjusted[e]
Age (years)	69.0 ±8.8	69.1 ±8.8	69.1 ±8.8	68.5 ±8.8	0.26		
Height (cm)	166.8 ±9.4	167.2 ±9.4	166.6 ±9.4	166.0 ±9.9	$4.4x10^{-6}$	$4.8x10^{-8}$[a]	0.72[a]
Weight (kg)	73.2 ±11.9	73.7 ±11.9	73.0 ±12.0	72.2 ±11.8	0.0006	0.13[b]	0.18[b]
BMI (kg/m^2)	26.3 ±3.7	26.4 ±3.7	26.2 ±3.7	26.2 ±3.9	0.32	0.32[a]	0.23[a]
LS BMD (g/cm^2)	1.09 ±0.2	1.09 ±0.2	1.09 ±0.2	1.08 ±0.2	0.47	0.78[c]	0.69[c]
FN BMD (g/cm^2)	0.87 ±0.1	0.87 ±0.1	0.87 ±0.1	0.86 ±0.1	0.16	0.91[c]	0.72[c]
LASA							
Age (years)	75.7 ±6.6	75.7 ±6.5	75.7 ±6.6	75.4 ±6.7	0.91		
Height (cm)	166.3 ±9.3	167.2 ±9.1	165.4 ±9.6	165.5 ±8.6	0.13	0.096[a]	NA
Weight (kg)	74.4 ±12.7	75.4 ±12.7	72.9 ±12.5	74.7 ±12.8	0.067	0.19[b]	NA
BMI (kg/m^2)	26.9 ±4.2	27.0 ±4.0	26.7 ±4.2	27.4 ±4.7	0.24	0.25[a]	NA
LS BMD (g/cm^2)	0.97 ±0.2	0.98 ±0.2	0.97 ±0.2	0.93 ±0.2	0.28	0.23[d]	NA
FN BMD (g/cm^2)	0. 69 ±0.1	0.71 ±0.1	0.69 ±0.1	0.70 ±0.1	0.37	0.84[d]	NA

[a] adjusted for age
[b] adjusted for age and height
[c] adjusted for age, height, weight and calcium intake
[d] adjusted for age, height and weight
[e] additionally adjusted for the principal components (PCA)
NA not applicable

LPH genotype and calcium intake

Data on nutrition intake were available only for subjects of the Rotterdam Study. As shown in Table 4, the T-13910C polymorphism is associated with dietary calcium intake, with C-homozygotes having lower dietary calcium intake (p trend = $3.0x10^{-5}$). C-homozygotes had, on average, a 93 mg/day (8.1%) lower calcium intake than T-homozygotes and a 62 mg/day (5.5%) lower calcium intake than heterozygotes. When we distinguished calcium intake from three different sources, we observed similar associations within the calcium intake from milk and from other dairy products groups but not in the non-dairy product group. All associations with calcium intake remained significant after adjustment for the PCs (Table 4). In the overall study popu-

lation, calcium intake and the bone-related endpoints studied were correlated; however, this was independent of lactose intolerance, as assessed by genotyping (data not shown). No associations between the T-13910C polymorphism and overall energy intake or protein intake were observed.

Table 4: Dietary calcium intake by LPH T-13910C genotype in the Rotterdam Study

	Total	TT	TC	CC	ANOVA		
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	p-value crude	p-value adjusted	p-value adjusted[c]
Dietary calcium intake (mg/day)							
Overall	1129 ±359	1151 ±352	1120 ±369	1058 ±339	$2.8x10^{-7}$	$8.8x10^{-7a}$	$3.0\ x10^{-5a}$
Milk	240 ±259	244 ±257	243 ±267	207 ±239	0.011	0.014[a]	0.032[a]
Milk-derivatives	256 ±203	268 ±209	252 ±197	214 ±191	$2.6x10^{-7}$	$1.7x10^{-7a}$	0.004[a]
Non-milk-derivatives	586 ±215	593 ±214	576 ±215	589 ±219	0.032	0.078[a]	0.05[a]
Total energy intake (MJ)	8.3 ±2.1	8.3 ±2.2	8.2 ±2.1	8.3 ±2.0	0.74	0.76[b]	0.24[b]
Total protein intake (g)	81.5 ±19	81.8 ±20	81.0 ±19	81.4 ±19	0.59	0.70[a]	0.64[a]

[a] adjusted for gender and age
[b] adjusted for gender, age and weight
[c] additionally adjusted for the principal components (PCs)

LPH genotype and serum calcium and vitamin D levels

In Table 5, data on serum calcium and vitamin D levels in both the Rotterdam Study and LASA are presented. In the Rotterdam Study, regression analyses showed a borderline significant trend towards lower total serum calcium levels for the C-variant (p = .09). Ionized calcium levels were lower in the C-homozygotes with evidence for an allele dose effect (p trend = .02). As in the Rotterdam Study, in LASA, serum calcium levels in C-homozygotes tended to be lower but regression analysis was not significant (p = .39). In the Rotterdam Study serum 25(OH) D_3 and $1,25(OH)_2D_3$ as well as serum 25(OH) D_3 in LASA study were not associated with the T-13910C polymorphism. Adjustment of PCs did not change the p values for both serum calcium and vitamin D levels associations

Interaction of the T-13910C polymorphism with the VDR

In the Rotterdam Study, interaction of the LPH T-13910C polymorphism with variants in the 3' UTR region of the VDR were tested. Of about 1.5% of the subjects either only the VDR block 5 haplotype 1 was available or only

the LPH polymorphism could be determined. This left 6047 subjects for interaction analyses between VDR and LPH.

Table 5: Serum total and ionized calcium levels and 25(OH)D and 1,25α$(OH)_2D_3$

	Total	TT	TC	CC	Linear Regression analysis	
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	p-value crude	p-value adjusted[a]
Rotterdam Study						
Total serum calcium (mmol/l)	2.39 ±0.15	2.39 ±0.15	2.38 ±0.15	2.38 ±0.15	0.12	0.09
Ionized serum calcium (mmol/l)	1.29 ±0.07	1.29 ±0.6	1.29 ±0.05	1.28 ±0.07	0.02	0.02
Serum 25(OH)D (nmol/l)	66.0 ±27.6	66.7 ±27.9	64.9 ±27.1	66.9 ±28.1	0.57	0.59
Serum 1,25α$(OH)_2D_3$ (pmol/l)	108.9 ±29.5	108.1 ±28.8	108.8 ±29.9	112.7 ±31.8	0.18	0.25
LASA						
Total serum calcium (mmol/l)	2.34 ±0.10	2.34 ±0.08	2.34 ±0.11	2.31 ±0.11	0.46	0.39
Serum 25(OH)D (nmol/l)	51.4 ±23.4	51.4 ±22.4	50.9 ±24.1	52.8 ±25.2	0.80	0.62

[a] adjusted for gender, age, height and weight

The VDR block 5 haplotype 1 consists of 10 SNPs and is identified by three tagging SNPs. The carriers of the block 5 haplotype 1 allele had an increased fracture risk [25]. Thus 70.5% of the subjects who were genotyped for LPH were carriers of the VDR block 5 haplotype 1. The association found between the T-13910C polymorphism and height in the VDR block 5 haplotype 1-carrying subgroup was due to stratification (Table 6). Furthermore, interaction analyses did not detect interactions between the two genotypes.

Discussion

This study demonstrates that some of the highly significant associations with the LPH T-13910C polymorphism, that is, height and vertebral area, were the result of population-stratification effects. However, dietary calcium intake and ionized calcium levels remained significant after adjusting for the population stratification. Analyses of the interaction between the LPH T-13910C polymorphism and the VDR block 5 haplotype 1 polymorphism did not show an interaction between these two polymorphisms.

In both the Rotterdam Study and LASA populations, genotype and allele frequencies did not follow Hardy-Weinberg equilibrium (HWE) proportions. Since the possibility of genotyping error is unlikely given the repeated geno-

typing and stringent quality control, ancestral selection at this locus may be underlying the observed HWE deviation. The possibility of ancestral selection affecting the LPH locus in our population is also supported by the strong attenuation of the effects on height after correction for principal components, which, in contrast to the global correction for genetic background assessed by the genomic inflation factor, takes into account more subtle and regional selection effects.

Table 6: Baseline characteristics by LPH T-13910C genotype stratified for VDR block 5 genotype in the Rotterdam Study

	VDR block 5 haplotype 1 non-carriers					VDR block 5 haplotype 1 carriers				
	LPH T-13910C genotype					LPH T-13910C genotype			ANOVA	
	TT	TC	CC	ANOVA	ANOVA PC adjusted	TT	TC	CC	p-value crude	p-value adjusted[e]
Age (years)[a]	68.9 ±8.7	69.1 ±8.8	69.0 ±8.8	0.74	0.70	69.1 ±8.8	69.0 ±8.8	68.5 ±8.8	0.32	0.63
Height (cm)[b]	166.9 ±9.3	167.5 ±9.6	166.7 ±8.8	0.23	0.28	167.3 ±9.5	166.4 ±9.2	166.4 ±10.0	0.002	0.24
Weight (kg)[c]	73.9 ±11.9	73.3 ±12.0	73.0 ±13.0	0.19	0.30	73.8 ±11.8	73.0 ±11.9	72.8 ±11.4	0.71	0.46
BMI (kg/m^2)[b]	26.5 ±3.8	26.1 ±3.6	26.2 ±3.6	0.14	0.20	26.3 ±3.7	26.3 ±3.7	26.4 ±4.0	0.92	0.68
LS BMD (g/cm^2)[d]	1.09 ±0.2	1.09 ±0.2	1.11 ±0.2	0.33	0.40	1.09 ±0.2	1.09 ±0.2	1.08 ±0.2	0.46	0.44
FN BMD (g/cm^2)[d]	0.86 ±0.1	0.87 ±0.1	0.88 ±0.1	0.44	0.44	0.87 ±0.1	0.87 ±0.1	0.86 ±0.1	0.85	0.91

[a] adjusted for gender
[b] adjusted for gender and age
[c] adjusted for gender, age and height
[d] adjusted for gender, age, height, weight and calcium intake
[e] adjusted for the principal component

This study shows an 8.1% lower calcium intake in C-homozygotes with evidence for an allele dose effect of the C-allele with an average of 1058 mg/day in the lactose-intolerant CC-group compared with 1120 mg/day in TC and 1151 mg/day in the TT group, that is, the lactose tolerant groups. Protein intake and energy intake were not different between the different genotypes. Most other studies that assessed the relationship between genetically defined lactose intolerance and calcium intake did show significantly lower calcium intake from milk but not lower overall dietary calcium intake (Table 1). In a group of young Finnish men, total dietary calcium intake was similar for the

three different genotypes, although calcium intake from milk and yogurt was lowest for the C-homozygotes [31]. These inconsistent findings could be explained at least in part by the low power of the studies. Another explanation for the observed discrepancies is that dietary habits between the various populations are different. In the study of Gugatschka and colleagues, calcium intake from milk and yogurt was only about 15% of the total daily calcium intake, whereas in our study calcium intake from yogurt and milk products, not including cheese, comprised about 50% of total daily calcium intake [8]. In addition, the overall calcium intake in several independent studies in Austria was approximately 600 g/day, which is about half the Dutch daily calcium intake [10].

Studies that have investigated the influence of lactose intolerance in relation to bone (Table 1) are conflicting and inconclusive. Differences in the prevalence, as well as social and cultural differences between populations, might have contributed to this, and selection criteria for the inclusion of participants may have diluted or enhanced associations. The sizes of these studies were small compared with our study, analyzing over 6000 individuals. An overall conclusion from both the Rotterdam Study and LASA is that the LPH T-13910C polymorphism is not associated with femoral neck and lumbar spine BMD or fracture risk. Although our study is a large population-based study, a limitation of our study is that we were not able to assess the severity of the lactose intolerance. Since complaints among lactose-intolerant persons vary, it is likely that persons with severe symptoms from lactose intolerance have even lower calcium intake levels and that in this subgroup there might be an effect on BMD and fracture risk.

None of the studies that assessed genetically defined lactose intolerance reported differences in height and / or weight [8, 11, 31, 32]. Our study clearly shows that the height and weight effects are probably not driven by the T-13910C polymorphism, but are more likely explained by differences in the genetic background of individuals and population stratification. Nonetheless, calcium and, more specifically, milk intake is suggested to influence height and weight, as demonstrated by Gugatschka and colleagues, who observed differences between milk and nonmilk drinkers in men on low calcium intake. The latter were 4 cm shorter and had a 5 kg lower body weight [8]. Milk-drinking habits therefore might influence bone and body dimensions on the basis of given genetic predisposition.

Fang and colleagues studied a comprehensive set of polymorphisms in the VDR gene and found the block 5 haplotype 1 to be associated with fractures

and body height [17, 25]. We studied whether carrying VDR block 5 haplotype 1 in LPH C-homozygotes would influence the observed associations because both genes are involved in intestinal calcium handling. A study by Obermayer-Pietsch and colleagues found intestinal calcium uptake to be impaired in lactose-intolerant subjects, when calcium was administered concomitantly with lactose [33]. Therefore, one might hypothesize that when expression of VDR is lower, such as might be the case for VDR block 5 haplotype 1 carriers and dietary calcium intake as well as uptake is lower in lactose-intolerant subjects, that is, C homozygotes for the T-13910C polymorphism, this might lead to interaction in association studies. However, the interaction term was not significant for the parameters analyzed in this study between the LPH T-13910C polymorphism and block 5 haplotype 1 ($p = .20$).

This is the first study to report pronounced differences in serum ionized calcium levels considering the T-13910C polymorphism. The physiologic mechanism behind this observed association regarding ionized calcium levels is unclear because ionized calcium levels are regulated primarily by sensing calcium levels in the parathyroid gland. However, the final regulation of proper calcium levels takes place via interplay among the parathyroid glands, kidneys, and intestines. Since parathyroid hormone (PTH) values are lacking, one can only speculate about possible explanations. One possibility might be an alteration of the setpoint for calcium within the parathyroids. Another explanation might be a subclinical secondary hyperparathyroid state owing to diminished intestinal uptake of calcium either by impaired intake of calcium or by impaired uptake, as suggested by Obermayer-Pietsch and colleagues [33]. Underlying this hypothesis is the observation by Di Stefano and colleagues that young adults suffering from a symptomatic lactose intolerance have low BMD values and increased PTH levels [2].

Conclusion

From this study we conclude that in a Dutch population with overall high calcium intake, the presence of the CC variant of the LPH polymorphism causing lactose intolerance is not associated with BMD and fracture risk. However, there is a clear association with dietary calcium intake and ionized calcium levels.

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Chapter 6

Novel Compound Heterozygous Mutations in the *CYP27B1* Gene Lead to Pseudovitamin D-Deficient Rickets

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Abstract

Background: Pseudovitamin D deficiency is the consequence of a genetic defect in the *CYP27B1* gene resulting in diminished or absent conversion of 25-hydrovitamin D_3 (25-(OH)D_3) into 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and leads to growth retardation and rickets, usually in the first two years of life.

Patient and methods: DNA obtained from human leucocytes from a patient suspected of pseudovitamin D deficiency and her healthy parents was sequenced for a genetic defect in the *CYP27B1* gene. *In silico* analyses on the mutations were performed using online available software. The 1α-hydroxylase activity of the patient, her parents and a sample derived from a mixed buffy coat of healthy blood donors was measured by culturing peripheral blood mononuclear cells with 25-(OH)D_3 and measuring 1,25-$(OH)_2D_3$ production.

Results: DNA sequencing of the patient suspected of pseudovitamin D deficiency revealed compound heterozygosity in the ***CYP27B1*** gene for a (c413G>T) mutation in exon 3 (R138L) and a (c1232G>A) mutation in exon 8 (C411Y). *In silico* analyses confirmed that mutations at these positions are probably damaging for the protein since the amino acids are situated in a highly conserved region. *In vitro* analyses showed a nearly absent 1α-hydroxylase activity in the patient compared to the healthy blood donors. Her healthy parents each of whom carried one of the mutations also had compromised conversion of 25-(OH)D_3 into 1,25-$(OH)_2D_3$ in peripheral blood mononuclear cells, being only marginally higher than the patient.

Conclusion: We discovered novel compound heterozygous mutations in the *CYP27B*1 gene in a young girl presenting with pseudovitamin D-deficient rickets, leading to severely decreased 1,25-$(OH)_2D_3$ production. Furthermore, both heterozygous parents showed a diminished 1α-hydroxylase activity.

Introduction

Vitamin D-dependent rickets has been classified in type 1 and type 2 rickets. Both types are subdivided in two forms, based on the underlying genetic defect. Type 1a, also called pseudovitamin D deficiency, comprises genetic defects in the *CYP27B1* gene, a member of the p450 cytochrome family located at position 13.1-13.3 on the long arm of chromosome 12 (OMIM *609506). This genetic defect results in defective or diminished hydroxylation of 25-hydroxyvitamin D_3 (25-(OH)D_3) into 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in the kidney. Physiologically, this leads to high 25-(OH)D_3 and low

1,25-$(OH)_2D_3$ levels, impaired calcium absorption in the intestines and elevated parathyroid hormone (PTH) resulting in hyperphosphaturia [1,2]. Pseudovitamin D defiency usually presents around 6-12 months after birth with severe skeletal disease, rachitic rosary, long bone bowing and enlarged metaphyses, failure to thrive, and growth retardation [3]. Biochemically, hypocalcaemia sometimes associated with tetany and mild-to-moderate hypophosphatemia can be found with elevated PTH levels. In this case report, we present a female patient with a severe form of pseudovitamin D-deficient rickets.

Patient & methods

Medical history and laboratory measurements

We studied a patient of Surinam-Hindustani origin who was admitted to the pediatrics ward at 11 months because of growth failure. On admission, she presented with remarkable thickness of the wrists and fusiform finger joints, bowing of the lower limbs, and a mild form of a rachitic rosary. Moreover, she suffered from hypotonia and anorexia, but there were no signs of motor development delay. None of her relatives had similar symptoms and her parents were healthy and unrelated.

Her laboratory values on admission are listed in Table 1, showing decreased serum calcium and phosphate levels with increased PTH, highly elevated AF levels, and decreased levels of 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$. An initial diagnosis of 1-alpha hydroxylase deficiency was considered.

Table 1: Laboratory measurements in the patient at 11 months of age.

Serum measurements	Pre-treatment values	After daily alfacalcidol administration	Reference values
Calcium (mmol/l)	1.79	2.29	2.10-2.60
Phosphate (mmol/l)	0.84	0.83	1.20-2.10
Alkaline phosphatase (U/l)	10,600	6,600	< 510
PTH (pmol/l)	1.14		<0.75
25-hydroxyvitamin D_3 (nmol/l)	12.5	117.5	50-120
1,25-dihydroxyvitamin D_3 (pmol/l)	7.5	177.5*	38-183

* After daily administration of 2000IU cholecalciferol.

We do not know why the 25-(OH)D_3 levels of the patient were so low, but we can hypothesize that this was due to anorexia in combination with a hyper-pigmented skin. It is also possible that the parents did not comply with the supplementation advice of 500IU daily of cholecalciferol for infants at that time [4].

On treatment with 1α-hydroxyvitamin D_3 (alfacalcidol 0.5 mcg/day) and phosphate (8.1 mmol/ per day), she developed normally. Menarche was at age 12. Her final height was 1.50 m, which she attained at age 13 with a weight of 36.2 kg.

At age 17, she was transferred form the children's out-patient clinic to internal medicine adult out-patient clinic. Laboratory results were not consistent with adequate treatment and patient was considered to be under treated (low serum calcium and phosphate, increased AF and PTH), which was thought to be due to decreased compliance. After restarting alfacalcidol and phosphate, patients' laboratory values normalized. At the age of 18, she developed autoimmune hypothyroidism, for which she was treated with levothyroxin. Phosphate supplementation was stopped at age 24 while alfacalcidol was continued.

In 2009 we invited her parents for further analysis. The father had a 25-(OH)D_3 level of 32 nmol/l with a 1,25-$(OH)_2D_3$ level of 62.9 pmol/l, while her mother had 70 nmol/l and 71.6 pmol/l levels of 25-(OH)D_3 and 1,25-$(OH)_2D_3$, respectively. Serum PTH level was 6.6 pmol/l in the father and 2.8 pmol/l in the mother (reference values PTH: 1.4-7.3 pmol/l, data not shown). For the clinical and genetic evaluation, the patient and her parents gave informed consent.

Strategies in Analyzing DNA and Assessing Mutations *In Silico* and *In Vitro*.

Methods on the collection of DNA and sequencing and details concerning the *in silico* and *in vitro* analyses can be found as a supplementary file.

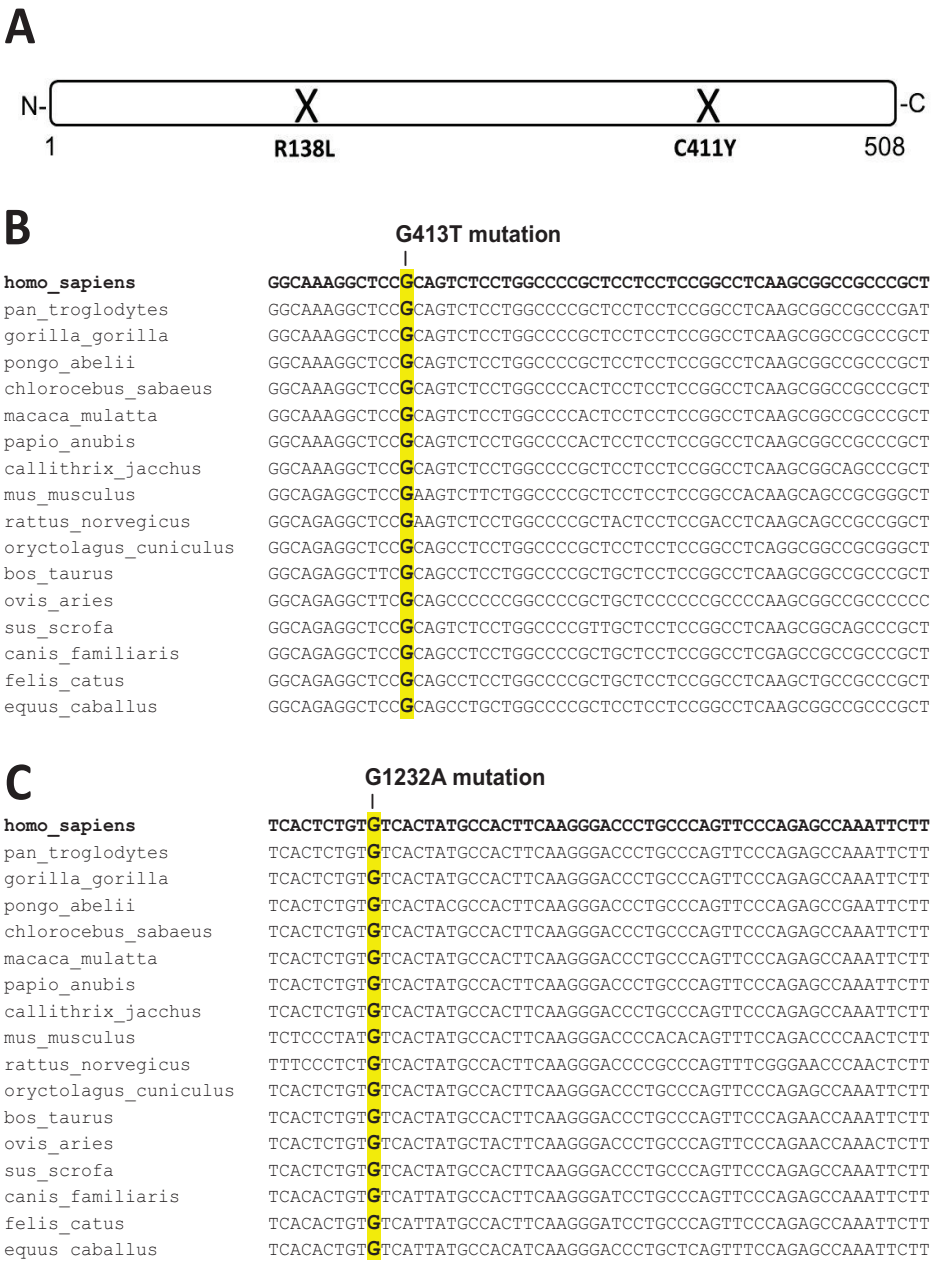
Results

Sequence Analyses

Sequence analyses of all exons revealed compound heterozygous mutations in the *CYP27B1* gene of the patient. The first is a c413G > T missense mutation found in exon 3 resulting in an arginine to leucine amino acid change at position 138. The second is a c1232G > A missense mutation in exon 8 leading to a cysteine to tyrosine amino acid change at position 411. The father was heterozygous carrier for the mutation in exon 3, whereas the mother was

heterozygous for the exon 8 mutation. The position of both mutations relative to the full protein have been schematically presented in Fig. 1a.

Figure 1: **G413T and G1232A mutation sites occur in evolutionary conserved regions** Structure of CYP27B1 with both mutations indicated relative to the full-length protein (A). Both mutations occur in evolutionary conserved regions across 17 orthologs (B and C)



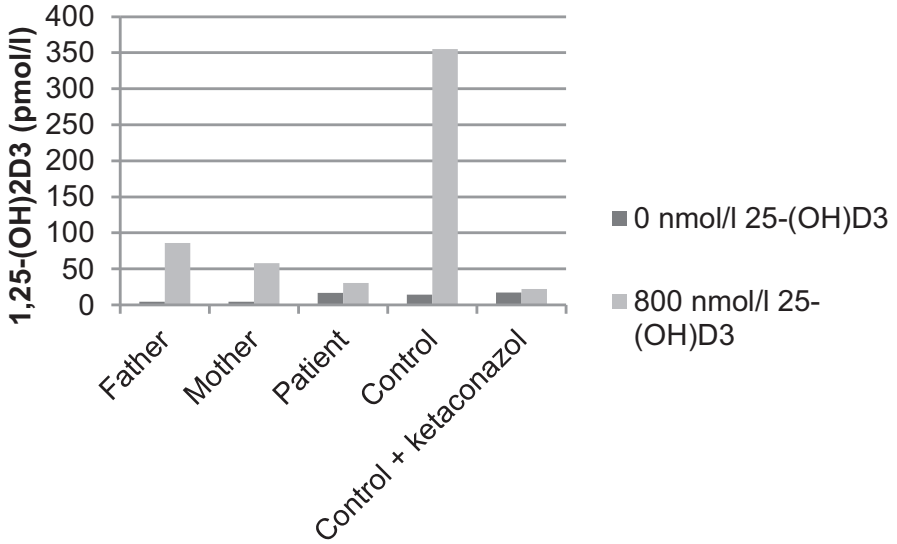
In silico Analyses

Using web-based prediction models, we assessed whether the two mutations affect the function of 1α-hydroxylase. Both the Polyphen 2 and Mutation Taster programs predict that the R138L and C411Y mutations are probably damaging to the protein or disease-causing, respectively. In addition, the Consurf software indicated that especially the R138L is highly conserved and predicted to be functional. These *in silico* findings are supported by comparison of the nucleotide sequences surrounding the orthologs of human 1α-hydroxylase across 17 species, indicating the evolutionary conservation of each mutation site (Fig. 1b, c). Extensive research in current databases did not yield SNPs at the chromosomal locations where both mutations were found.

1α-Hydroxylase Activity Assay in PBMCs

In order to assess the 1α-hydroxylase activity of the patient and the parents compared to healthy controls, we measured 1,25-$(OH)_2D_3$ levels in PBMC cultures supplemented with 800 nmol of 25-$(OH)D_3$ for a 24-h period (Fig. 2). After 24 h, 1,25-$(OH)_2D_3$ levels in the supernatant increased up to 355 pmol/l in the controls. The levels of 1,25-$(OH)_2D_3$ from the patient's PBMC cultures were more then tenfold lower compared to the controls and increased up to 30.7 pmol/l. Both parents showed higher 1,25-$(OH)_2D_3$ levels after administration of 25-$(OH)D_3$ compared to the patient but these did not reach values of the control, i.e. 86.1 pmol/l for the father and 58.1 pmol/l for the mother.

Figure 2: 1,25-$(OH)_2D_3$ levels after 24 hour incubation with 0 and 800 nmol/l 25-$(OH)D_3$ in PBMCs.



Discussion

In this study, we found novel compound heterozygous mutations in a patient who presented with severe signs of pseudovitamin D deficiency at a young age. Sequence analyses revealed novel compound heterozygous mutations in exons 3 and 8 (R138L/C411Y) of ***CYP27B1***, the 1α-hydroxylase gene, which led to diminished enzyme activity as shown by minimal conversion of 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$ in PBMC cultures.

The first to describe mutations responsible for the pseudovitamin D deficiency were Kitanaka *et al.* [5]. They found four homozygous mutations in four different patients, two of them in exon 2, one in exon 6, and one in exon 7 of the 1α-hydroxylase gene. Fu *et al.* found compound heterozygote mutations in the keratinocytes of a girl with pseudovitamin D deficiency [6]. In 2007, Kim *et al*. reviewed 44 patients with a genetic defect in the gene encoding the 1α-hydroxylase enzyme and added data on ten new patients where they confirmed pseudovitamin D deficiency using sequencing. As for the latter ten patients, five carried a homozygous mutation, four patients had compound heterozygous mutations, and in one patient only one heterozygous mutation was found [7]. An overview of the mutations in the *CYP27B1* gene was published in 2012 [8,9]. From 2012 onwards, eight novel mutations have been described to date [10-13] which we added in Table 2 to this paper including our own mutations. In total, we are aware of 34 missense mutations, four nonsense mutations, 11 deletions, 3 insertions, 2 deletion-insertions and 6 splice-site mutations.

Table 2: Mutations found in literature from 2012

Nucleotide change	Amino acid Change	Exon
1294C>A	R432S	Exon 8 [13]
1079C>A	S360	[10]
195T>G	Frameshift codon 65	Intron 1 [10]
1022-1037del16	Frameshift T341Rfs*5	Exon 6 [12]
1215+2T>A	L380Afs*57	Intron 7 [12]
934_935delAC	T312Rfs*19	Exon 5 [12]
1165C>T	R389C	Exon 7 [11]
1375C>T	R459C	Exon 8 [11]
413G > T	Arg138Leu	Exon 3 (this study)
1232G > A	Cys411Tyr	Exon 8 (this study)

To our knowledge both mutations found in our patient have not been described previously. Both are located in proximity of an α-helix structure and likely compromise the structure of the protein as was predicted by commonly used software programs. Unfortunately, no crystal structure for the CYP27B1 enzyme is available and therefore not much is known about how the mutations affect structure and how this is related to the function of the enzyme.

Most functionality analyses to scrutinize protein defects are performed using cells transfected with the discovered mutation, or by obtaining keratinocytes from patients. Besides the kidney, keratinocytes have long been thought to be the only site of vitamin D metabolism acting in an autocrine/paracrine manner [6,14]. However, 1α-hydoxylase activity has been found in many tissues since then, and van Driel et al. showed that human peripheral blood mononuclear cells express *CYP27B1* and exert 1α-hydroxylase activity when cells are stimulated with RANKL[15]. This prompted us to study our patient in a straightforward functionality assay, using easy-to-obtain peripheral blood mononuclear cells. We have demonstrated that this assay is a reliable assay to assess *CYP27B1* functionality and that the mutation found in our patient led to a more than tenfold reduction in enzyme activity compared to control. Interestingly, while the parents had no vitamin D-related health problems, the *in vitro* functionality assay showed strongly reduced conversion of 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ compared to healthy control cultures. Smith et al. found normal values in the heterozygous parents of the patients when compared to controls after stimulation of macrophages with INF-γ, but with considerable variation [16]. Currently, it seems there is not a clear-cut correlation between the mutation and residual enzyme activity and the clinical phenotype. This is supported by the discrepancy in observations by Kitanaka et al. [17] and Wang et al. [18] on patients with mild clinical phenotypes. However, we cannot definitively rule out a genotype-phenotype relation as various enzymatic assays and functional readouts including transfection assays and natural cells have been used reflecting different sensitivities. There is still the possibility that a genotype-phenotype relation does exist since mild cases might not come to the attention of the doctor or might not be diagnosed as being pseudovitamin D deficiency. Since the autocrine/paracrine role of 1α-hydroxylase in tissues besides keratinocytes and kidney is only partly understood [14], we cannot completely rule out that there may be subclinical effects leading to symptoms later in life.

In conclusion, we describe a female patient with two novel compound heterozygous mutations (R138L/C411Y) in *CYP27B1,* leading to vitamin D-dependent rickets. We demonstrated that the patient had a diminished 1α-hydroxylase activity as shown by minimal conversion of 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$ in PBMC cultures, which occurred to a lesser extent in both the parents.

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Supplemental Material and Methods

Strategies in analyzing DNA and assessing found mutations in silico and in vitro.

Methods on the collection of DNA and sequencing and details on the in silico and in vitro analyses can be found as a supplementary file.

Collection of DNA and sequencing

After informed consent, genomic DNA was isolated from peripheral blood leucocytes of the patient and her parents, using a magnetic particle manipulator according to the manufacturer's protocol (Agowa, Berlin, Germany).

Primers for the different exons of the *CYP27B1* gene were previously described by Smith et al.[9] and PCR was performed, using an Amplitaq Gold kit (Applied Biosystems). The PCR products corresponding to the different exons of the *CYP27B1* gene were sequenced (BaseClear, Leiden, the Netherlands) and analyzed, using freely available software (Chromas lite, http://www.technelysium.com.au/chromas_lite.html).

***In silico* analyses**

To evaluate mutations at protein level *in silico*, we used the web-based programs Mutation Taster (http://www.mutationtaster.org/) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph/) to predict if the mutation is disease causing or damaging, respectively. In addition, Consurf (http://consurf.tau.ac.il/) was utilized to determine whether the mutation is situated in a conserved area of the protein, indicating its importance for protein function.

PBMC cultures

Details on PBMC collection have been described previously by van der Eerden *et al.*[10]. In short, following collection of PBMCs from the patient and her parents, cells were seeded in a density of 500,000 cells/well in a 96-well plate and incubated for 4 hours. Then, cells were washed once with 1x Phosphate-Buffered Saline and culture medium was supplemented with 800 nmol/l 25-(OH)D_3, generously provided by Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). After 24 hours, the conditioned medium was collected and stored at –20ºC until the samples were used for 1,25-$(OH)_2D_3$ measurement, using a ^{125}I-radioimmunoassay (IDS, Boldon, UK). As healthy control, PBMC samples derived from a blood bank buffy coat of pooled mixed healthy donors (Sanquin, Amsterdam, the Netherlands) were incubated with the same concentration of 25-(OH)D_3. Ketaconozole (10^{-4}M) served as a negative control in the blood bank-derived PBMC cultures.

Chapter 7

Osteoclastogenic capacity of peripheral blood mononuclear cells is not different between women with and without osteoporosis

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Abstract

Introduction: Peripheral Blood Mononuclear Cells (PBMCs) have been extensively used as a culture model to generate osteoclasts *in vitro*. The aim of this study was to assess the osteoclastogenic potential of PBMCs derived from postmenopausal women with longstanding osteoporosis and compare this with PBMCs from healthy controls.

Material and Methods: We selected from the population-based Rotterdam Study 82 participants of which 43 were diagnosed with osteoporosis (T-score below -2.5 at the lumbar spine) and the presence of at least 1 fracture and 29 healthy controls (T-score above 1; no fracture). PBMCs were differentiated into osteoclasts, and both differentiation capacity and activity were measured. Total RNA was obtained to assess gene expression of osteoclast markers. Deoxypyridinoline (DPD) was measured in plasma as a marker for bone resorption, *in vivo*.

Results: Neither the number of osteoclasts nor cathepsin K (CTSK) and dendritic cell-specific transmembrane protein (TM7SF4) gene expression was significantly different between both groups. There was also no significant difference in resorption pit area and plasma DPD levels. Stratification by fracture type into a group with vertebral, non-vertebral, and both vertebral and non-vertebral fractures showed no difference in osteoclast formation or osteoclastic bone resorption. However, plasma DPD, but not the RNA expression markers, was significantly lower in the group of subjects with vertebral fractures and those with vertebral and non-vertebral fractures compared to the healthy controls. No differences in osteoclastogenesis, osteoclastic resorption, and plasma DPD levels were detected also after exclusion of past or present users of bisphosphonates and glucocorticoids. Stratification into high and low DPD levels showed higher osteoclastogenesis and more osteoclastic bone resorption in the high DPD group compared to the low DPD levels within the group of osteoporotic subjects.

Conclusion: This study showed no difference in PBMC osteoclastogenic capacity and activity between women with and without osteoporosis and at least one previous fracture, who were on average 29.5 years after menopause, suggesting that there is no difference in circulating osteoclast precursors. Although we cannot exclude that circulating precursors may behave differently at the bone site, it is possible that long after menopause a more stable phase of bone turnover is reached compared to earlier after the start of menopause in which differences in circulating osteoclast precursors and osteoclastogenic potential are more prominent.

Introduction

Osteoporosis is an age-related disorder with a marked morbidity and mortality. In The Netherlands, the overall prevalence of osteoporosis is 16.1/1000 for women and 1.9/1000 for men [1]. The incidence of vertebral fractures in The Netherlands above 55 years is 14.7/1000 person years for women and 5.9/1000 person years for men [2]. Osteoporosis results from a shift in the balanced bone remodeling towards increased bone resorption over bone formation, resulting in bone loss and higher risk of fractures.

Human peripheral blood mononuclear cells (PBMCs) are a good source for the generation of osteoclasts [3, 4]. A study by D'Amelio and coworkers showed an increase in spontaneous osteoclastogenesis and lacunar resorption area in women with postmenopausal osteoporosis compared to postmenopausal women without osteoporosis but found no difference in osteoclast number and lacunar resorption when cultures were stimulated with growth factors [4]. A study by Jevon *et. al.*, did not find a difference in osteoclast formation in PBMC cultures between osteoporotic women and healthy women but reported an increased activity when PBMC's were co-cultured with the rat osteosarcoma cells UMR106 [5], a cell-line with osteoblast characteristics [6].

The aim of our study was to investigate whether elder women, who were on average 25 years after menopause, with established osteoporosis and at least one fracture after the age of 55 years, demonstrate increased PBMC-derived osteoclast formation and increased osteoclast activity *in vitro* compared to age- and years since menopause-matched healthy controls.

Material and Methods

Selection and inclusion procedure

We selected women from the Rotterdam Study, a cohort of elderly Caucasian subjects from which long-term follow-up data are available on bone mineral density (BMD) and fractures. Details on study design and rationale have been described extensively [7]. For this study we selected women diagnosed with osteoporosis (osteoporotic group) and women with normal BMD (control group) that served as a control. The osteoporotic group included women who sustained either a vertebral fracture, clinical or radiological, or an non-vertebral fracture defined as a fracture of the hip, wrist, or humerus, and a BMD T-score level ≤ -2.5 SD at the lumbar spine (LS), measured at their 4th visit at the center, which took place between January 2002 and July 2004. We chose LS BMD since it consists mostly of trabecular bone, which is con-

sidered to be more metabolically active compared to cortical bone [8]. This group had also a highly significant reduced femoral neck BMD compared to the women that served as a control (Table 2). Fractures in the osteoporotic group had occurred between 1991 and 2001, excluding prevalent vertebral fractures at baseline. At the 4th visit, none of the participants had reported a new fracture. Details of the assessment of incident non-vertebral fractures and vertebral fractures have been described in detail previously [9]. For the control group we selected age- and years-since menopause-matched women who never experienced non-vertebral or vertebral fractures and with a LS BMD T-score between +1 and +2.5 SD as measured at the fourth visit between January 2002 and July 2004. Furthermore, their femoral neck BMD T-score had to be above -1 SD. From this study, 171 potential participants were invited, of which 47% agreed to participate. We included 43 participants in the osteoporotic group and 29 in the control group. Reasons for potential participants not to participate varied from not being interested to not being able to come to the research facility due to deteriorating health. The Rotterdam Study design is approved by the Medical Ethics Committee and by the review committee of the Ministry of Health, Welfare and Sports. The approval has been renewed every 5 years. The study design and rationale of the subselection of the Rotterdam Study for the study described in this paper was approved by the Medical Ethics Committee (number NL20078.0780.07). Written informed consent was given by all participants.

Intake and blood sampling

Participants were invited for a single visit at the research centre between the 4th of August and the 15th of October of 2008, where they underwent a short questionnaire, concerning their mobility, medical history, and smoking, and venous blood collection. Medication use was recorded and for 67 of the 72 participants this could be verified with the participants' general practitioner and pharmacy database (Medicom®). For 4 participants, data on medication use was lacking either due to the participant being absent in Medicom or not being able to inform us about medicine use. Data on nutritional supplement intake was recorded on basis of recollection. 7 ml of plasma and 30 ml of heparinized blood was collected from each participant for the measurement of plasma markers and collection of PBMCs for osteoclast cultures, respectively. All visits took place in the morning in order to isolate the PBMCs in the afternoon. Between collection of blood and isolation of PBMCs the samples were stored at room temperature.

PBMC collection

PBMCs from the participants were obtained from heparinized blood by Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. In the afternoon after returning from the research centre, cells were resuspended in α-MEM culture medium (Gibco, Life Technologies, Breda, The Netherlands) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands), 250 ng/ml amphotericin B (Sigma, St.Louis, MO, USA), 1.8 mM $CaCl_2$ (Elitech Benelux, Spankeren, The Netherlands), and 15% (vol/vol) heat-inactivated fetal calf serum (FCS; Life Technologies). Cells were counted on a CASY TT cell counter (Innovatis AG, Reutlingen, Germany). From the total amount, 2 million cells were used for RNA isolation (time point 0; see below) and stored in Trizol Reagent (Gibco BRL, Life Technologies) at –20ºC. The remainder of the cells was divided over 2 vials, frozen and stored in liquid nitrogen until further use.

PBMC cultures

In order to minimize differences between culture conditions and handling the cells, the same two persons performed PBMC cultures of a maximum of 12 randomly selected samples in the morning. Cells were thawed and taken up in α-MEM culture medium that was made in 1 batch to be used in all experiments. To minimize inter-participant variability, PBMCs from 12 participants were cultured per day.

The cells were counted and seeded in a density of 200,000 cells/well either on plastic or bovine cortical bone discs in 96-wells plates (Corning, # 3595). Per subject a minimum of 12 wells for Coommassie staining, 12 wells for RNA analyses and 8 to 16 wells for TRAP staining were used, depending on the total amount of cells available for culture. As mentioned above, cells from the participants were randomized over the plates.

Following seeding, cells were incubated for 8 hours and then rinsed once with PBS to remove non-adherent cells. Next, culture medium was added containing 25 ng/ml human macrophage-colony stimulating factor (M-CSF; R&D Systems, Abingdone, UK). From day 4 onward, cells were cultured in the presence of both M-CSF and 30 ng/ml human receptor-activated NF-κB ligand (RANKL; PeproTech, London, UK). In order to minimize differences between test conditions medium and growth factors, the amounts of each product necessary for the experiment was calculated before the start of the culture experiment and were batched in advance to establish identical cul-

ture conditions. Half of the media/well was refreshed twice a week until day 21 of culture.

TRAP staining assay

At day 21 of culture, cells on plastic were fixed with 70% ethanol and stained, using a Tartrate Resistance Acid Phosphatase (TRAP) leucocyte kit (Sigma, St.Louis, MO, USA) as described previously by van der Eerden *et al*. [10]. Per well 5 photos were taken to limit the effect of unequal development of osteoclasts over the plate and to minimize variation between wells. The number of osteoclasts and nuclei per osteoclast were counted by an independent observer who was blinded to the data concerning the experimental layout, using online available Image J software (https://imagej.nih.gov/ij/).

Resorption pit assay

In order to assess bone resorption, cells cultured on bovine cortical bone discs were stopped at day 21 and lysed with water. After this the discs were stained with filtered Coomassie brilliant blue (Phastgel Blue R; Amersham Pharmacia Biotech, Roosendaal, The Netherlands) for 5 seconds as described previously [10]. Bone resorption was quantified by measuring the mean resorbed bone area, using Image J software, which was performed by an independent observer, who was blinded towards the data.

DPD assay

Total Deoxypyridinoline (DPD) cross-links were determined in the plasma, taken at the single visit at the research centre, from all participants by performing an ELISA (MicroVue total DPD, Quidel Corporation, San Diego, USA) according to the manufacturer's protocol.

RNA isolation, cDNA synthesis and Q-PCR

Cells were collected direct after PBMC collection (time point 0, see above) and after 21 days of culture on bone discs. RNA isolation, cDNA synthesis and Q-PCR have been carried out as described before by Bruedigam et al. [11]. Oligonucleotide primer pairs, all being either on exon boundaries or spanning at least one intron, were purchased from Sigma-Aldrich (Table 1). Gene expression was normalized against *GAPDH* according to the equation $2^{-\Delta Ct}$ (ΔCt = Ct of gene of interest minus Ct of housekeeping gene).

Table 1: Primer sequences used

Primers	Forward primer (5'- 3')	Reverse primer (5'- 3')
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
CTSK	TGCCCACACTTTGCTGCCGA	GCAGCAGAACCTTGAGCCCCC
TM7SF4	AAGCAGCCGCTGGGAGAAGT	TTTTCAGGACTGGAAGCCAGAAATGAA

Statistics

Spearman's non-parametric bivariate correlation model was used for assessing associations between variables. A One-Way Anova was used to test for group differences, and if homogeneity of variances could not be assumed, we performed the Welch test. Post-hoc test were included in the One-Way Anova analyses in order to assess associations between the type of fracture sustained and the observed variables that were not specified *a priori*. Statistics were performed using the statistical program SPSS 19.

Results

Study group characteristics are shown in Table 2. Participants were on average 78.9 years old. For 17 controls and 31 osteoporotic participants data on age of menopause was known. Controls were on average 27.9 years after menopause and osteoporotic subjects were 30.8 years after menopause, which was not significantly different (data not shown). Age was not significantly different between the groups, but body height, weight, and BMI were significantly lower in the osteoporotic group compared to the control group.

In the osteoporotic group 20 subjects had sustained a clinical vertebral fracture, 15 subjects sustained a non-vertebral fracture e.g. hip, wrist, or humerus, and 8 subjects had sustained both a vertebral fracture and a non-vertebral fracture.

Osteoclast formation and function is not different between osteoporotic patients and controls

When comparing participants with osteoporosis to controls we found no differences in PBMC-derived osteoclast formation, the amount of single-, bi-, multi-nucleated, or total osteoclasts *in vitro* or in the mean resorbed bone area (Table 3 and Figure 1). The mRNA expression levels at day 21 of cathepsin K (*CTSK*, an enzymatic bone resorption marker) and dendritic cell-specific transmembrane protein (*TM7SF4*, a cell surface receptor important for the fusion of mononucleated osteoclasts) were not significantly different

between subjects with and without osteoporosis. Moreover, DPD, a plasma marker of bone resorption, was not significantly different between both groups. Additional adjustments for weight and BMI did not alter any of the results.

Table 2: Baseline characteristics

	Controls (CI)	**Osteoporosis (CI)**	**P-value**
Number	29	43	
Age (years)	77.23 (75.32-79.12)	79.51 (78.00-81.02)	0.06
Height (cm)	164.76 (160.59-164.85)	161.89 (157.75-160.95)	0.011
Weight (kg)	78.00 (74.00-82.01)	64.36 (61.89-66.83)	$3.46x10^{-9}$ *
BMI (kg/m^2)	29.60 (27.77-31.44)	25.41 (24.33-26.48)	$6.00x10^{-5}$
Lumbar spine BMD (g/cm^2)	1.37 (1.35-1.39)	0.81 (0.79-0.83)	$6.87x10^{-52}$ *
Femoral neck BMD (g/cm^2)	1.00 (0.96-1.03)	0.73 (0.70-0.77)	$2.99X10^{-16}$

*Welch test due to significant inhomogeneity of variances. Shown is mean with 95% confidence intervals between parentheses.

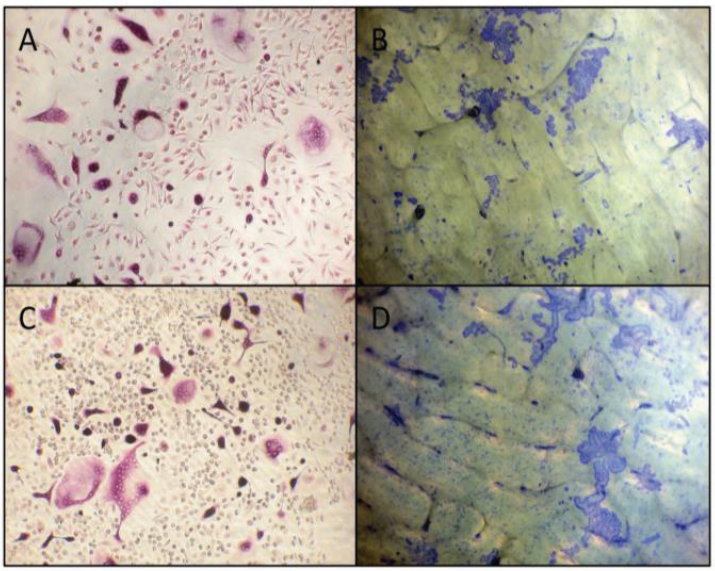

Figure 1: No differences in PBMC-derived osteoclast differentiation and resorption between healthy and osteoportic subjects. Panel A and B represents TRAP stained osteoclasts from control subject (A) and from an osteoporotic subject (C). Panels B and D represent resorption pits stained with Coommassie Blue staining from a controls subject (B) and from and osteoporotic subject (D) (Magnification 100X).

Table 3: Osteoclast formation and resorption parameters

	Controls (CI)	Osteoporosis (CI)	P-value
Mononuclear osteoclasts (#)	14.30 (7.29-21.30)	12.31 (9.52-15.09)	0.54
Binuclear osteoclasts (#)	5.44 (3.34-7.53)	8.58 (1.55-15.62)	0.47
Multinuclear osteoclasts (#)	7.71 (3.68-11.75)	7.79 (4.57-11.00)	0.98
Total number of osteoclasts (#)	27.44 (16.90-37.99)	28.66 (18.20-39.12)	0.87
Mean resorption area (mm^2)	0.011 (0.005-0.015)	0.012 (0.006-0.018)	0.77
CTSK expression (rel. to GAPDH)	3.40 (2.48-4.32)	3.10 (2.00-4.21)	0.70
TM7SF4 expression (rel. to GAPDH)	0.07 (0.04-0.10)	0.10 (0.07-0.13)	0.16[a]
DPD (nmol/l)	10.99 (10.25-11.73)	10.86 (10.86-13.90)	0.94

[a]Welch test due to significant inhomogeneity of variances. Shown is mean with 95% confidence intervals between parentheses.

In the combined study population of osteoporotic participants and controls the osteoclast formation ability of the PBMCs *in vitro* was significantly correlated with the mean resorbed bone area on the bone slices, with the highest correlation found for multinucleated TRAP positive cells ($R = 0.56$; $p = 4.1 \times 10^{-8}$) (Table 4). The mRNA expression of *CTSK* and *TM7SF4* in these cultures was significantly correlated with the osteoclast formation ability ($R = 0.65$, $p = 3.9 \times 10^{-11}$ and $R = -0.63$; $p = 3.1 \times 10^{-10}$, respectively) and mean resorbed bone area ($R = 0.75$, $p = 1.3 \times 10^{-15}$ and $R = -0.55$; $p = 1.4 \times 10^{-7}$, respectively). However, we found no correlation between plasma DPD and osteoclast formation, mean resorbed bone area (Table 4), and gene expression of *CTSK* and *TM7SF4 (data not shown)*. Lumbar spine BMD and femoral neck BMD were not correlated with osteoclast formation ability, resorbed bone area, and *CTSK* and *TM7SF4* gene expression. However, DPD was significantly and positively correlated with lumbar spine and femoral neck BMD in combined osteoporotic and control population ($R = 0.52$, $p = 1.1 \times 10^{-6}$ and $R = 0.49$; $p = 1.7 \times 10^{-5}$, respectively) (Table 4).

To assess whether there was a difference in osteoclast formation and function depending on DPD levels we stratified the osteoporotic group as well as the control group into high and low DPD levels based on the median value for that group. As shown in Table 5, osteoporotic subjects with a DPD level below the median had significantly lower mean resorbed bone area and a significantly reduced number of osteoclasts compared to osteoporotic subjects with high DPD levels (mean resorbed bone area 0.020 versus 0.004, $p = 0.009$ and number of multiple nucleated osteoclast 11.94 versus 4.19, $p = 0.017$). This

remained significant after exclusion of the two subjects with very high DPD levels (data not shown). Stratification of controls above and below their median DPD level did not show any significant differences in osteoclast formation ability, osteoclastic bone resorption ability or *CTSK* and *TM7SF4* mRNA expression levels.

Table 4: Correlation coefficients (R) for the number of osteoclast, bone resorption area, gene expression, BMD and DPD for the combined osteoporotic and control group.

	Multi-nucleated osteoclasts (#)		**Mean resorption area (mm^2)**		**Lumbar spine BMD (g/cm^2)**		**Femoral neck BMD (g/cm^2)**	
	R	P-value	R	P-value	R	P-value	R	P-value
Multinucleated osteoclasts (#)	-	-	0.57	$1.6x10^{-7}$	0.02	0.90	0.08	0.54
Mean resorption area (mm^2)	0.57	$1.6x10^{-7}$	-	-	0.11	0.36	0.19	0.12
CTSK expression	0.64	$2.9x10^{-9}$	0.75	$3.8x10^{-14}$	0.13	0.30	0.22	0.07
TM7SF4 expression	-0.61	$1.1x10^{-8}$	-0.57	$2.3x10^{-7}$	-0.11	0.34	-0.82	0.51
DPD (nmol/l)	0.19	0.12	0.24	0.05	0.48	$2.9x10^{-5}$	0.54	$4.0x10^{-6}$

Table 5: Study parameters, stratified according to DPD above and below the median in osteoporotic subjects and in controls

	Osteoporotic group			**Control group**		
	DPD > median	DPD < median	P-anova	DPD > median	DPD < median	P-anova
Number individuals	21	20		12	16	
Mononuclear osteoclasts (#)	14.77 (10.32-19.21)	10.63 (6.94-14.31)	0.14	17.22 (9.26-25.18)	12.10 (5.21-19.00)	0.57
Binuclear osteoclasts (#)	14.51 (0.03-28.99)	3.14 (1.51-4.78)	0.12[a]	4.12 (-6.54-14.77)	5.90 (-3.32-15.13)	0.68
Multinuclear osteoclasts (#)	11.94 (6.79-17.08)	4.19 (0.32-8.06)	0.017[a]	7.40 1.31013.48)	6.95 (-1.68-12.22)	0.92
Total number of osteoclasts (#)	41.21 (21.75-60.67)	17.93 (3.67-25.61)	0.028	28.73 (10.23-47.24)	24.95 (8.93-40.98)	0.87
Mean resorption area (mm^2)	0.012 (0.009-0.031)	0.004 (0.001-0.007)	0.009[a]	0.009 (-0.001-0.019)	0.012 (0.004-0.021)	0.82
CTSK expression	3.99 (2.10-5.88)	2.36 (0.99-3.73)	0.16	3.51 (1.63-5.38)	3.29 (1.67-4.92)	0.96
TM7SF4 expression	0.06 (0.04-0.10)	0.13 (0.07-0.19)	0.04[a]	0.06 (0.00-0.11)	0.08 (0.04-0.13)	0.44

[a]Welch test due to significant inhomogeneity of variances. Shown is mean with 95% confidence intervals between parentheses

Fracture type

To evaluate whether there was an association between fracture type and osteoclast formation and resorption, we stratified the osteoporotic group according to different types of fractures (vertebral fractures, non-vertebral fractures, and a group of subjects with both vertebral and non-vertebral fracture). These analyses did not reveal differences between the control group and any of the fracture groups (Table 6). Although the difference in total number of osteoclasts between controls and subjects with vertebral fractures was considerable, with controls having higher values, it was not statistically significant.

Table 6: Study parameters, stratified according to fracture type

	Control	Vertebral fractures	Non-vertebral fractures	Vertebral and non-vertebral fractures	P-value (anova)
	29	15	20	8	
Mononuclear osteoclasts (#)	14.30 (7.29-21.30)	10.88 (7.16-14.60)	13.19 (8.58-17.81)	12.76 (3.25-22.27)	0.89
Binuclear osteoclasts (#)	5.44 (3.34-7.53)	2.71 (1.26-4.16)	12.17 (-2.56-26.89)	10.64 (-4.20-25.48)	0.39
Multinuclear osteoclasts (#)	7.71 (3.68-11.75)	3.80 (0.52-7.08)	11.19 (5.33-17.06)	6.75 (-1.24-14.74)	0.22 *
Total number of osteoclasts (#)	27.44 (16.90-37.99)	17.39 (10.11-26.66)	36.52 (15.81-57.23)	30.15 (6.22-54.08)	0.36 *
Mean resorption area (mm^2)	0.011 (0.005-0.015)	0.008 (0.000-0.017)	0.014 (0.000-0.024)	0.014 (0.000-0.028)	0.73
CTSK expression	3.40 (2.48-4.32)	2.08 (1.13-3.04)	3.25 (1.72-4.78)	4.66 (-0.45-9.78))	0.30 *
TM7SF4 expression	0.07 (0.04-0.10)	0.11 (0.051-0.160)	0.08 (0.032-0.119)	0.15 (0.021-0.270)	0.18 *
DPD (nmol/l)	10.99 (10.25-11.73)	8.68 (8.08-9.28)	13.31 (6.62-19.99)	8.85 (7.61-10.09)	0.003 *

*Welch test due to significant inhomogeneity of variances. Shown is mean with 95% confidence intervals between parentheses.

Influence of potentially confounding medication

In order to exclude possible influence of bisphosphonate use in this study we performed additional analyses in a subgroup of participants who never used bisphosphonates. From all 72 participants we could retrieve whether they had been given prescriptions of bisphosphonates. From 68 out of these 72 we had data on current bisphosphonate use. One person stopped taking bisphosphonates 2 weeks prior to the blood sampling and was therefore scored as a

bisphosphonate user. The data lacking with regard to current bisphosphonate use concerned 3 participants in the control group and 1 participant in the osteoporosis group. In the osteoporotic group, 14 participants had never used and 12 had stopped using bisphosphonates more than 8 months prior to the sample extraction. A total of 26 participants in the osteoporotic group were not on any bisphosphonate prescription around the time of participation. None of the control subjects used bisphosphonates when participating in this study. One control subject had been prescribed bisphosphonates until two years before participating, due to glucocorticoid use.

No significant difference between the osteoporotic and control group was detected concerning number of TRAP positive single, bi-nucleated, or multi-nucleated cells, mean resorbed bone area and *CTSK* and *TM7SF4* expression after exclusion of ever-users of bisphosphonates (data not shown). Furthermore, serum DPD levels did not differ significantly between the osteoporotic and the control group either after additional adjustment for bisphosphonate use or after exclusion of users of bisphosphonates (data not shown).

Discussion

The current study demonstrates no difference in osteoclastogenic potential from circulating precursors in women with and without osteoporosis 29.5 years after cessation of regular menses. In *ex vivo* osteoclastogenic cultures of PBMCs no differences in osteoclast formation and bone resorption activity was observed. A possible explanation might be the age of the participants at the time of inclusion in the study. It is known that there is an accelerated bone loss shortly after menopause that is mainly due to decreased estrogen levels leading to uncoupling of bone formation and bone resorption [12]. Sometime after this initial phase, osteoclast activity may slow down to become undistinguishable from healthy controls.

Two smaller studies on *in vitro* osteoclastogenesis and osteoclastic bone resorption found similar results. The first was performed in 2003 by Jevon *et al*. whom found no differences in TRAP-positive cells and bone resorption between 11 osteoporotic participants and 12 controls [5]. In agreement with this, D' Amelio *et al.* did not observe differences in osteoclastogenesis and bone resorption when comparing 18 postmenopausal osteoporotic women with 15 healthy controls after stimulation of PBMCs with M-CSF and RANKL [4]. Unfortunately, both studies lack data on participant characteristics concerning age, height and weight, and years since menopause. Fur-

thermore, they selected the participants on basis of their T-score in contrast to our study where we selected on basis of the presence of at least one non-vertebral or vertebral fracture in addition to a low T-score. Our study has been conducted in a population of elderly women who have been extensively followed and from whom a detailed history on fractures exists.

We sampled at an average age of 78.7 years and therefore potential differences in the marked increased postmenopausal osteoclastogenesis as the result of rapid decrease in estrogen levels may have been missed. Supporting this theory is the observation that the bone turnover marker DPD was not significantly different between controls and osteoporotic subjects. However, given the fact that low estrogen levels persist at older age despite the observed absence of increased bone turnover markers in this study, fracture risk may not be associated with increased bone turnover, since fracture risk increases with age, well after the onset of menopause [2]. Moreover we showed that DPD was significantly positively correlated with lumbar spine BMD and femoral neck BMD. Nevertheless, in a longitudinal study on bone turnover markers and the risk of fracture in 75-year-old women it was found that elevated bone resorption markers CTX and S-TRACP5b were associated with fracture risk [13]. In contrast to our observation of osteoporotic subjects having similar DPD levels as their age-matched controls without osteoporosis, Garnero showed that bone resorption markers remain elevated even in late menopause up to 40 years after menopause [14]. However, other studies have suggested that increased bone turnover in late menopause as measured by bone resorption markers was due to vitamin D deficiency and/or hypocalcaemia or secondary hyperparathyroidism [15-17]. Unfortunately, we did not have data on calcium, vitamin D, or PTH levels, during sampling so we could not investigate whether these values were normal in our subjects.

We found an opposite correlation of *TM7SF4* and *CTSK* gene expression with osteoclast formation and resorbed bone area, being negative and positive, respectively. Both correlations can be explained when considering the function of TM7SF4 and CTSK. TM7SF4 is necessary for cell fusion and multinucleation of mononuclear osteoclasts [18]. Hence, reduced expression in combination with increased resorbed bone area is indicative for fully matured osteoclasts that have developed beyond the stage of fusion. CTSK is a lysosomal cysteine protease, and catabolizes elastin, collagen and gelatin, which is necessary for osteoclasts to resorb the organic fraction of bone [19]. Indeed, the positive correlation implicates that mature, resorbing osteoclasts are associated with higher CTSK expression. These findings suggest that our

culture system is a valid tool to correlate human osteoclast function with gene expression.

Considering the culture system used, several discrepancies were noted compared to other studies, where PBMCs were used for the generation of osteoclasts. First of all, we used PBMCs whereas other studies have used CD14+ cells for the generation of osteoclasts [20]. Furthermore, one can argue about the addition of M-CSF and RANKL in these PBMC cultures. The study by d'Amelio *et al.* showed enhanced PBMC-derived osteoclastogenesis in osteoporotic patients compared to controls without using M-CSF and RANKL, but as expected, in this study the overall numbers of osteoclasts and the amount of resorption for both groups were much lower compared to when M-CSF and RANKL were administered [4]. We cannot exclude other potential reasons for our results and those by others such as the assay itself and the fact that circulating precursors may not be different in their activation state, but potentially *in situ* at local sites e.g. around recent fractures they may be. To date, a standard curve of osteoclastogenic potential and capacity does not exist, but if there are means to develop an alternative way to assess the osteoclastogenic capacity in an easy and robust manner, it would constitute an interesting and applicable approach.

Stratification of osteoporotic subjects into those with high and low DPD levels showed differences in *in vitro* osteoclastogenesis and osteoclastic bone resorption. The subjects with lower DPD levels might reflect a late stage of osteoporosis with lower bone metabolic activity as is the case in senile osteoporosis [21] or osteoporosis with more uncoupling of bone formation and resorption. Unfortunately we had no availability of markers of bone formation to support this theory. It could be that osteoporosis patients with high PBMC osteoclastogenesis and DPD levels above the median represent a group with higher risk of fracture compared to the osteoporosis patients with DPD levels below the median. Considering the fact that the drug of first choice in the treatment of osteoporosis is the administration of anti-resorptive therapy, one can argue whether giving anti-resorptive therapy in the subgroup of patients with low bone resorption would be the most beneficial form of therapy or that an anabolic agent would be more effective in reducing fracture risk in this particular subgroup.

Fractures and bisphosphonate use

To our knowledge, this is the first study to assess fracture type in osteoporotic patients in relation to *in vitro* osteoclast formation and bone resorption. We only found DPD levels to be significantly lower in persons with vertebral

fractures and with vertebral and osteoporotic fractures compared to the healthy controls, which could not be attributed to bisphosphonate use. In a previous study conducted by d'Amelio *et al.* the influence of risedronate intake on *in vitro* osteoclastogenesis and osteoclast activity from human PBMCs was studied. They found a 68% reduction of osteoclast formation and activity with risedronate treatment but not following calcium and vitamin D administration [23]. A study conducted to assess the effect of alendronate on BMD and bone turnover markers showed that after discontinuation of alendronate, a gradual decline in BMD of about 3% was observed and a gradual rise in bone turnover markers over 5 years to levels still slightly below pretreatment levels [24]. In our study, we found no difference in osteoclastogenesis, osteoclastic bone resorption and DPD serum markers between the osteoporotic and the control group, even after the exclusion of bisphosphonate users, but numbers of individuals might be limited to detect small differences.

In conclusion, in this study no differences in PBMC-derived osteoclastogenesis and osteoclastic bone resorption were observed between elderly women with osteoporosis and healthy controls, suggesting that there is no difference in circulating osteoclast precursors. Although we cannot exclude that circulating precursors may behave differently at the bone site, it is conceivable that long after menopause a more stable phase of bone turnover is reached compared to shortly following the initiation of menopause in which differences in circulating osteoclast precursors and osteoclastogenic potential are more prominent. To gain more insight in osteoclastogenesis and lacunar bone resorption in osteoporosis, we suggest obtaining PBMCs from women shortly after menopause, when bone resorption activity is high due to rapidly declining estrogen levels. Finally, large prospective data and a more robust assay would be needed to evaluate whether the assessment of osteoclastogenesis and bone resorption *in vitro* will aid to the identification of subjects who are at higher risk for future fracture and thus in a better selection of those who may benefit most from a treatment with anti-resorptive therapy.

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Chapter 8

Lifelong challenge of calcium homeostasis in male mice lacking TRPV5 leads to changes in bone and calcium metabolism

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Abstract

Background: Trpv5 plays an important role in calcium (Ca^{2+}) homeostasis, among others by mediating renal calcium reabsorption. Accordingly, *Trpv5* deficiency strongly stresses calcium (Ca^{2+}) homeostasis in order to maintain stable serum Ca^{2+}. Previous studies have shown that *Trpv5*$^{-/-}$ mice display vitamin D resistance with aging at the level of the kidneys and intestines with hypercalciuria. Therefore, we addressed the impact of lifelong challenge of calcium homeostasis on the bone phenotype of these mice.

Material and methods: *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ male mice were studied at 10, 52 and 78 weeks of age. Serum Ca^{2+}, PTH and vitamin D were assessed to evaluate homeostasis. Femurs were studied for microarchitecture, degree of mineralization and frailty by means of microcomputed tomography, quantitative backscattered electron imaging and 3-point-bending. Osteoclast formation and function were assessed *ex vivo*. Osteoclast precursors were evaluated by flowcytometric analysis.

Results: Aging significantly increased serum $1,25(OH)_2D_3$ and PTH levels in both genotypes but they were more elevated in *Trpv5*$^{-/-}$ mice, whereas serum Ca^{2+} was not affected by age or genotype. Age-related changes in trabecular and cortical bone mass were accelerated in *Trpv5*$^{-/-}$ mice, including reduced trabecular and cortical bone thickness as well as reduced bone mineralization. No effect of *Trpv5* deficiency on bone strength was observed. In 78-week-old mice no differences were observed between the genotypes regarding urinary deoxypyridinoline, osteoclast number, differentiation and activity.

Conclusion: Life-long challenge of Ca^{2+} homeostasis as present in *Trpv5*$^{-/-}$ mice causes accelerated bone aging and a low cortical and trabecular bone mass phenotype. The phenotype of the *Trpv5*$^{-/-}$ mice suggests that maintenance of adequate circulatory Ca^{2+} levels in patients with disturbances in Ca^{2+} homeostasis should be a priority in order to prevent bone loss at older age. The *Trpv5*-deficient mouse appears to be a suitable model for lifelong challenge of calcium homeostasis and its consequences for bone metabolism.

Introduction

Maintenance of adequate Ca^{2+} levels is of crucial importance for many physiological processes in the body including neuronal excitability, muscle contraction and bone formation. Bone is the major site of Ca^{2+} storage in the body, and formation and mineralization by osteoblasts as well as osteoclastic bone resorption, contribute to the maintenance of Ca^{2+} equilibrium in the circulation. Serum Ca^{2+} is tightly regulated through the concerted interactions of

kidneys, intestines and bone. Transcellular Ca^{2+} (re)absorption is an important process in maintaining Ca^{2+} balance by these tissues [1, 2].

Previously, we published on the phenotype of mice lacking the epithelial Ca^{2+} channel Trpv5 (*Trpv5*$^{-/-}$) [3]. TRPV5 is a Ca^{2+}-selective transient receptor potential channel that is expressed in renal epithelial cells and crucial for reabsorption of calcium. In *Trpv5*$^{-/-}$ mice, besides hypercalciuria, intestinal Ca^{2+} hyperabsorption takes place by upregulation of the close homolog of Trpv5, Trpv6. This process is impaired when $1,25(OH)_2D_3$ bioactivity is disturbed as shown in double knockout mice for TRPV5 and 1α-hydroxylase (synthesizes $1,25(OH)_2D_3$) and by treatment of *Trpv5*$^{-/-}$ mice with a vitamin D receptor antagonist, ZK191784 [4, 5].

A detailed study on bone in these mice revealed that Trpv5 has a direct role in bone [6]. *Trpv5*$^{-/-}$ mice display an aberrant bone phenotype, including reduced cortical and trabecular bone thickness. Within bone, TRPV5 appears to be expressed by osteoclasts exclusively at the site where bone resorption takes place. Despite enhanced osteoclastogenesis, both *in vivo* and *in vitro*, bone resorption is seriously disturbed in mice lacking *Trpv5*, indicating that TRPV5 is required for proper osteoclast function.

It is known that increasing age is accompanied by changes in Ca^{2+} homeostasis, including reduced Ca^{2+} absorption from the diet, reduced vitamin D availability, which predisposes older persons to disorders related to Ca^{2+} homeostasis, particularly secondary hyperparathyroidism and osteoporosis [7, 8]. In addition, the capacity of $1,25(OH)_2D_3$ to stimulate intestinal Ca^{2+} absorption declines with age, whereas circulating levels of PTH rise with age in rats and humans [9, 10]. Moreover, age-related increase in PTH levels may play an important role in changes in bone remodeling. Bone loss occurs universally with aging, leading to a reduction in bone mass and strength eventually leading to bone fragility and increased risk of osteoporotic fractures in the elderly [7, 11].

We previously demonstrated that compared to wildtype (*Trpv5*$^{+/+}$) mice, both renal Ca^{2+} reabsorption and intestinal Ca^{2+} absorption were reduced during aging in *Trpv5*$^{-/-}$ mice, of which the latter was associated with *Trpv6* expression [12]. Moreover, elevated vitamin D receptor protein levels observed in the intestine in older mice are indicative for vitamin D resistance.

In this study we aimed to investigate the bone phenotype of aging *Trpv5*$^{+/+}$ and Trpv5$^{-/-}$ mice by detailed analyses of serum and urine parameters related to calcium homeostasis and bone resorption, bone microarchitecture, mineralization and strength *in vivo*. Moreover, bone marrow cultures from long

bones were performed to assess osteoblast and osteoclast differentiation in 78-week-old *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice. Finally, the impact of aging on calcium homeostasis and bone-related gene expression was examined in femurs and bone marrow cultures from both genotypes.

Results

Serum 1,25(OH)$_2$D$_3$ and PTH are age-dependently elevated in Trpv5$^{-/-}$ mice

No difference in serum Ca^{2+} levels were observed between *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice at all three ages (Table 1). Serum Ca^{2+} in the oldest age group (78 weeks) was measured with a different calcium assay, which hampers direct comparison between the younger 2 age cohorts with the 78-week-old mice.

Table 1: Serum and urine measurements in *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice during aging

	10 weeks		52 weeks		78 weeks	
	***Trpv5*$^{+/+}$**	***Trpv5*$^{-/-}$**	***Trpv5*$^{+/+}$**	***Trpv5*$^{-/-}$**	***Trpv5*$^{+/+}$**	***Trpv5*$^{-/-}$**
	mean ± sem	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*
Serum						
Calcium (mmol/l) * #	2.76 ± 0.02	2.84 ± 0.02	2.71 ± 0.04	2.79 ± 0.04	2.02 ± 0.27	1.70 ± 0.29
1,25(OH)$_2$D$_3$ (pmol/l)	121 ± 24	686 ± 77 [b]	542 ± 41	518 ± 108	474 ± 50 [c]	1321 ± 133 [b,d]
Parathyroid hormone (pg/ml) #	9.9 ± 1.9	22.6 ± 6.0	30.7 ± 6.2	100 ± 20.6 [a]	84.8 ± 21.9 [c]	60.2 ± 18.1

[a] p<0.05 vs *Trpv5*$^{+/+}$ mice of same age, [b] p<0.001 vs *Trpv5*$^{+/+}$ mice of same age. [c] P<0.05 for age trend in *Trpv5*$^{+/+}$ mice. [d] p<0.05 for age trend in *Trpv5*$^{-/-}$ mice.

*Levels at 78 weeks of age were measured by different assay compared to 10 and 52 weeks

#Published previously by Abel et al.[1]

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However, using the same assay, previous measurements in an aging cohort of wild type mice up to 2 years of age yielded similar Ca^{2+} levels compared to the 78-week-old mice in this study (Supplementary table 2). In *Trpv5*$^{+/+}$ mice, 1,25(OH)$_2$D$_3$ (pmol/l) and PTH (pg/ml) levels increased significantly with aging (Figure 1 and Table 1). In *Trpv5*$^{-/-}$ mice a similar age-related increase in serum 1,25(OH)$_2$D$_3$ was observed but serum 1,25(OH)$_2$D$_3$ levels were significantly higher in *Trpv5*$^{-/-}$ compared to their *Trpv5*$^{+/+}$ littermates at 10 and

78 weeks of age. PTH level in *Trpv5*$^{-/-}$ mice increased with age but was only significantly higher at 52 weeks age compared to *Trpv5*$^{+/+}$ mice. In 10-week-old *Trpv5*$^{-/-}$ mice, both PTH and $1,25(OH)_2D_3$ were at a level that is not reached before 52 weeks of age in *Trpv5*$^{+/+}$ littermates (Figure 1 and Table 1).

Trabecular and cortical bone mass are reduced in TRPV5$^{-/-}$ mice

In *Trpv5*$^{+/+}$ mice, trabecular bone thickness (Figure 2A), trabecular spacing and structure model index (SMI) were increased in older mice, whereas trabecular number and connectivity density demonstrated an age-related decline (Supplementary table 3). *Trpv5*$^{-/-}$ mice showed similar age-related changes as *Trpv5*$^{+/+}$ mice in the trabecular compartment although their trabecular bone thickness increased less pronounced during aging resulting in significantly lower bone mass in 52- and 78-week-old mice compared to their *Trpv5*$^{+/+}$ littermates (Figure 2A).

Figure 1

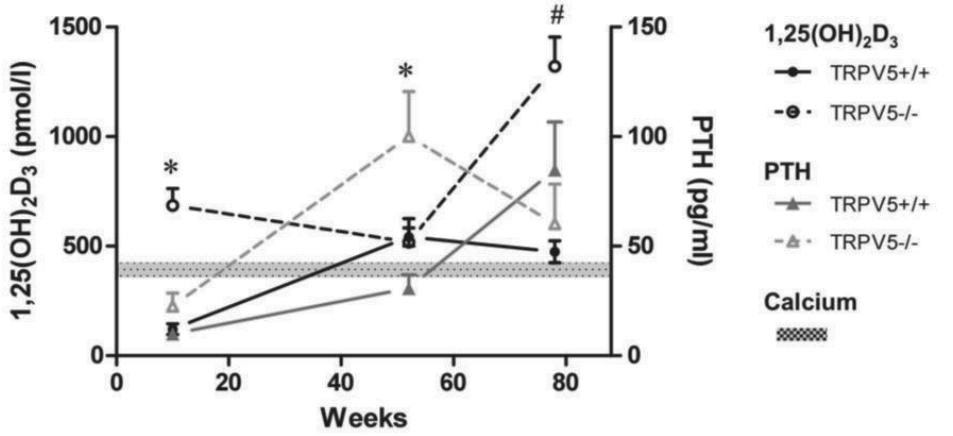

Figure 1: TRPV5 deficiency leads to elevated serum $1,25(OH)_2D_3$ and PTH levels
Depicted is an illustration of temporal changes in serum levels of hormones involved in calcium homeostasis. Based on the measured serum calcium levels in the 10 and 52-week old animals and calcium measured of 78-week-old male mice in this and a previous study, we have depicted calcium to be constant irrespective of age and genotype (black dotted line, not representing an actual value). Both $1,25(OH)_2D_3$ (triangle symbols) and PTH (round symbols) levels are elevated in 10-week-old *Trpv5*$^{-/-}$ (open symbols) compared to *Trpv5*$^{+/+}$ mice (closed symbols). In fact, the serum levels for both hormones in 10-week-old TRPV5 deficient mice are similar to those in 52-week-old *Trpv5*$^{+/+}$ mice. The data presented are not longitudinal and hence should not be presented as a line graph but they serve solely as an illustration for temporal serum changes in mice lacking Trpv5. We emphasized this by disconnecting the lines from the actual measurements.

Within the cortical bone compartment, an age-related decrease in cortical thickness (Figure 2B) was seen in *Trpv5*$^{+/+}$ mice, while a stable cortical bone volume was maintained throughout life. Consistent with these findings, we found the endocortical volume (i.e. marrow cavity volume) to be increased with aging (Figure 2C-D). TRPV5 deficiency led to similar age-related changes, but cortical thickness (Figure 2B) and cortical volume (Supplementary table 3) were reduced at all ages compared to the *Trpv5*$^{+/+}$ mice. At 78 weeks of age, both endocortical volume and cortical porosity were significantly greater in the *Trpv5*$^{-/-}$ mice compared to their non-deficient littermates (Figures 2C and 2D, respectively). Other cortical parameters, such as moment of inertia and perimeter also increased during aging but they were not different between the genotypes (Supplementary table 3).

Mineralization of the trabecular structure is reduced in Trpv5$^{-/-}$ mice

Tibial quantitative backscattered electron (qBEI) imaging showed in trabecular bone an age-related increase in the average and most abundant mineralization densities (CaMean and CaPeak, respectively) and an age-related reduction in the areas undergoing primary mineralization (CaLow) in wildtype mice (Figures 3A, B and D). The heterogeneity of mineralization (CaWidth) did not alter during aging in *Trpv5*$^{+/+}$ mice (Figure 3C). *Trpv5*$^{-/-}$ mice demonstrated age-related changes for all qBEI parameters measured being positively correlated with age for CaMean, CaPeak and CaWidth (Figures 3A, B and C, respectively), whereas CaLow was negatively correlated (Figure 3D). Both CaMean and CaPeak were reduced in the younger *Trpv5*$^{-/-}$ mice, being significant at 52 weeks and 10 weeks of age versus *Trpv5*$^{+/+}$ mice, respectively (Figures 3A and 3B, respectively). In the metaphyseal cortical bone, all parameters changed with age in both genotypes (Supplementary table 4). In contrast to the trabecular data, in cortical bone no difference for any of the parameters at any age was observed between the genotypes (Supplementary table 4). Both in *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice, cortical CaMean, CaPeak and CaWidth increased with age, whereas CaLow was negatively correlated (Supplementary table 4). In the cortices of both genotypes osteocyte lacunae number and size were assessed. No difference was found for both parameters when corrected for cortical bone area (Supplementary figure 1).

Bone resorption in Trpv5$^{+/+}$ and Trpv5$^{-/-}$ bones during aging

Bone resorption as assessed by urinary DPD was similar between the *Trpv5*$^{+/+}$ mice age groups (Figure 4A). In contrast, *Trpv5*$^{-/-}$ mice demonstrated an age-related increase in DPD levels. At 10 and 52 weeks of age, *Trpv5*$^{-/-}$

mice had significantly lower DPD levels compared to *Trpv5*$^{+/+}$ mice but at 78 weeks of age, these were similar in both genotypes (Figure 4A). Osteoclast numbers and surface in femoral bone sections of 78-week-old were reduced in *Trpv5*$^{-/-}$ mice but this did not reach statistical significance (Figures 4B and 4C, respectively). In bone marrow cultures derived from 78-week-old mice, osteoclast numbers generated from *Trpv5*$^{-/-}$ precursor cells were significantly lower (Figure 4D), but this caused no difference in in vitro bone resorption (Figures 4E). Frequencies of bone marrow populations containing osteoclast precursors (i.e. immature blasts, myeloid blasts, and monocytes [13] as evaluated by flowcytometry were not different between *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice (Supplementary table 5). The only different cell population in the bone marrow pool was the lymphoid precursor cells, which were significantly lower in the *Trpv5*$^{-/-}$ mice at 78 weeks.

Trpv5 deficiency does not affect bone strength

Three-point-bending tests were performed on a subset of femurs from male 78-week-old *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice. There were no differences in maximum load, stiffness or energy of the femurs between the genotypes (Supplementary figures 2A-C). Moreover, Young's modulus, a measure for elasticity of the bone, was unaffected (Supplementary figure 2D).

Old Trpv5$^{-/-}$ and Trpv5$^{+/+}$ mice show differences in femoral gene expression patterns

Next, we generated femoral bone gene expression profiles for both genotypes at 10, 52 and 78 weeks of age by focusing at genes involved in calcium transport and homeostasis, osteoclast function, bone metabolism and phosphate homeostasis. *Calcium transport and homeostasis: Trpv6* mRNA expression in femurs of *Trpv5*$^{+/+}$ mice was lower in the 78-week-old animals compared to younger age groups, though not significant (Figure 5A). Calcium sensing receptor (*Casr)* and vitamin D receptor (*Vdr*) mRNA expression were negatively and positively correlated with age in *Trpv5*$^{+/+}$ mice, respectively (Figure 5B and C, respectively). Other genes involved in transcellular Ca^{2+} transport, i.e. calbindin-D_{9K} (*S100g*), sodium/calcium exchanger 1 (*Ncx1*) and plasma membrane calcium ATPase 1 (*Atp2b1*) did not change during aging of *Trpv5*$^{+/+}$ mice (Supplementary table 6). Comparing *Trpv5*$^{-/-}$ and *Trpv5*$^{+/+}$ mice, no significant differences were observed for any of the genes related to calcium homeostasis at any time point. Besides reduced *Vdr* expression at 52 and 78 weeks of age (Figure 5C) and increased *Ncx1* expression at 78 weeks of age (Supplementary table 6).

Figure 2

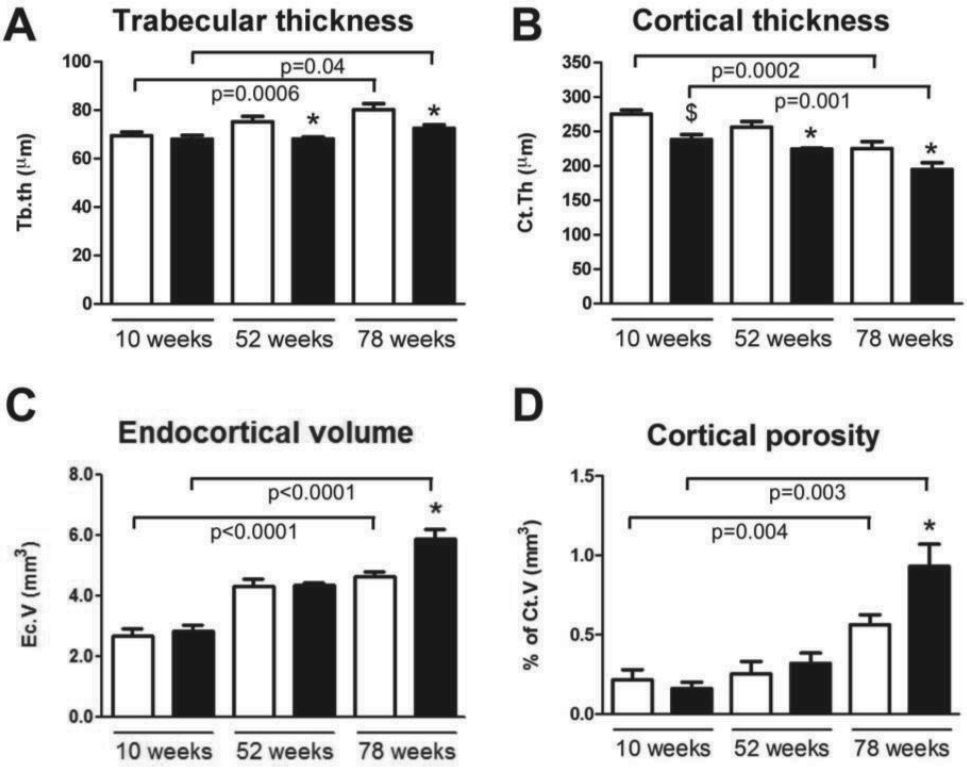

Figure 2: TRPV5 deficiency leads to reduced bone thickness
Bone microarchitectural parameters from male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice at 10, 52 and 78 weeks of age were determined by µCT analysis (n=5-11). A) trabecular thickness in the metaphysis, B) cortical thickness, C) endocortical volume and D) cortical porosity in the diaphysis. Data are presented as means ± SEM. * p<0.05 versus *Trpv5*$^{+/+}$. $^{\#}$ p<0.01 vs *Trpv5*$^{+/+}$. $^{\$}$ p<0.001 vs *Trpv5*$^{+/+}$. Significant aging effects for a genotype are indicated by horizontal lines.

Osteoclast function: The mRNA expression of the osteoclast marker genes for tartrate-resistant acid phosphatase (*Acp5*), cathepsin K (*Ctsk*) and the calcitonin receptor (*Ctr*) was negatively correlated with age in both genotypes (Figures 5D-E and Supplementary table 6). All osteoclast marker genes assessed were slightly but consistently elevated in the *Trpv5*$^{-/-}$ compared to *Trpv5*$^{+/+}$ mice (Figures 5D-E and Supplementary table 6), but only reached significance for *Acp5* and *Ctsk*. *Bone metabolism and phosphate homeostasis:* We also assessed several genes associated with bone metabolism and phosphate homeostasis. Among them, phosphate regulating endopeptidase homolog, X-linked (*Phex*), osteopontin (*Spp1*) and sclerostin (*Sost*)) showed reduced mRNA expression with aging in *Trpv5*$^{+/+}$ mice (Figure 5F and Supplementary table 6). Ankylosis, progressive homolog (Ank), Fibroblast growth factor

23 (Fgf23) and Klotho (Kl) were not modulated in an age-related manner in these mice (Supplementary table 6). As for the *Trpv5*$^{+/+}$ mice, *Phex*, *Spp1* and *Sost* expression showed a negative correlation with age in *Trpv5*$^{-/-}$ mice (Figure 5F and Supplementary table 5). Only *Phex* expression at 78 weeks of age was significantly different in *Trpv5*$^{-/-}$ mice compared to *Trpv5*$^{+/+}$ mice (Figure 5F). Overall, the *Trpv5*$^{-/-}$ mice showed similar age-related trends as did the *Trpv5*$^{+/+}$ mice but for some genes significantly higher expression of bone-related genes was observed at 78 weeks of age compared to the *Trpv5*$^{+/+}$ mice. In bone marrow-derived osteoblasts and osteoclasts, all marker genes studied were expressed at lower levels in the *Trpv5*$^{-/-}$ mice, but none of these differences reached significance (Supplementary table 7).

Figure 3

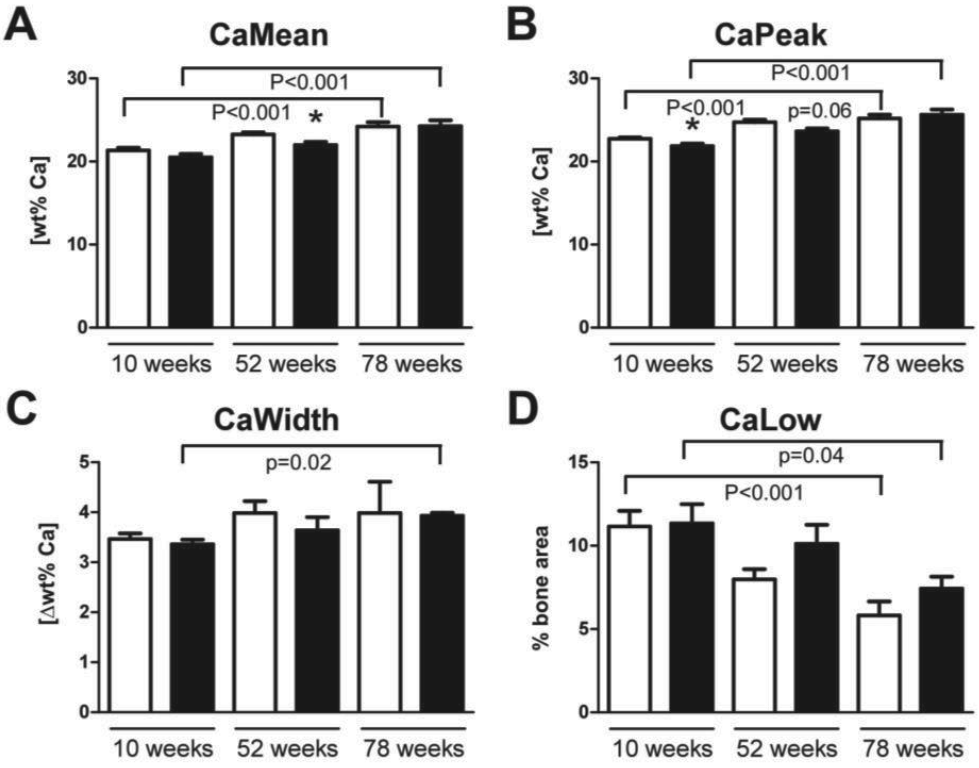

Figure 3: Bone mineralization is affected in *Trpv5*$^{-/-}$ mice
Using quantitative backscattered electron imaging, the tibial BMDD was assessed from male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice at 10, 52 and 78 weeks of age (n=3-6). Parameters were measured in the metaphyseal spongious bone compartment with respect to A) CaPeak, B) CaMean, C) CaWidth (all expressed as units of [wt%Ca]) and D) CaLow (expressed as percentage bone area). Values are presented as mean ± SEM. * p<0.05 versus *Trpv5*$^{+/+}$. $^{\#}$ p<0.01 vs *Trpv5*$^{+/+}$. $^{\$}$ p<0.001 vs *Trpv5*$^{+/+}$. Significant aging effects for a genotype are indicated by horizontal lines.

Figure 4

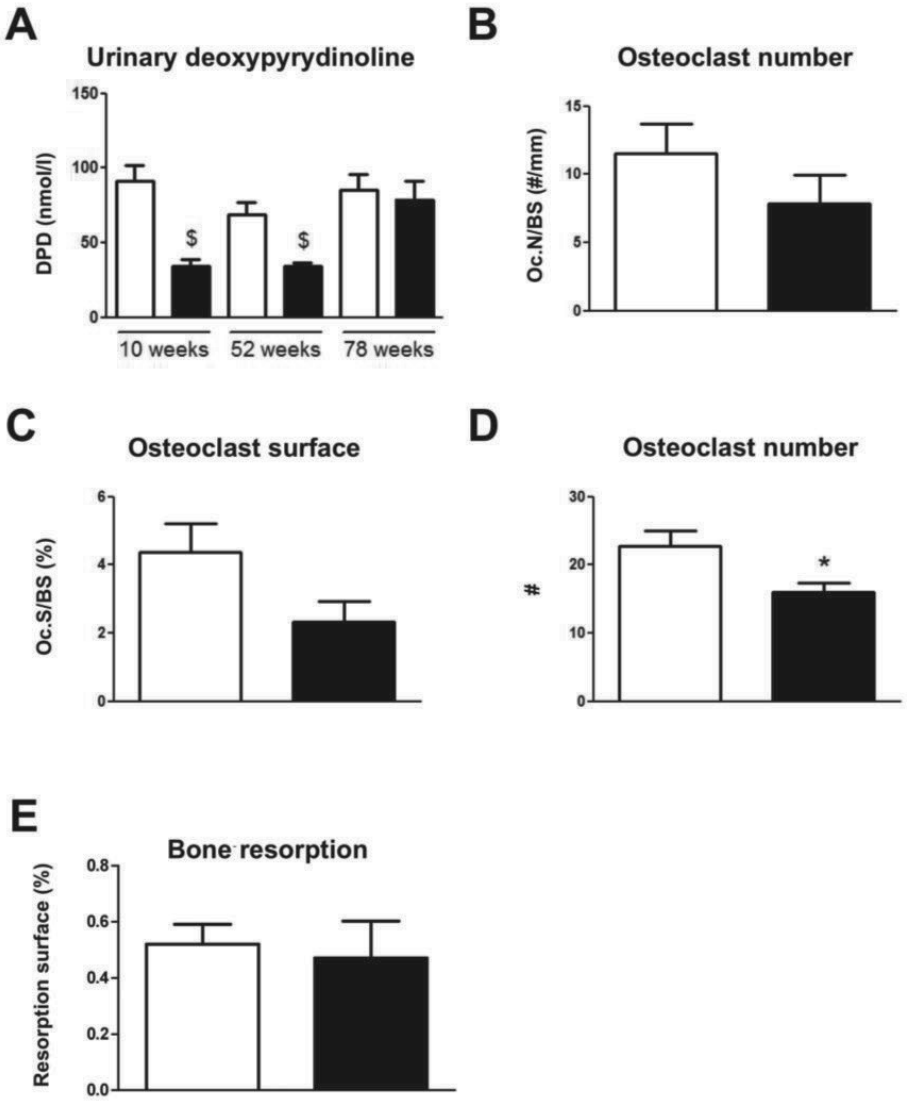

Figure 4: Bone resorption is reduced in *Trpv5*$^{-/-}$ mice until 52 weeks of age
A) Urinary DPD measurement in 10, 52 and 78-week-old male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice. B) osteoclast number and C) osteoclast surface per bone surface in TRAP-stained femoral bone section of 78-week-old male *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice. D) osteoclast number and E) osteoclast surface assessed in bone marrow-derived osteoclast cultures from 78-week-old male *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice. Values are presented as mean ± SEM. * $p<0.05$ versus *Trpv5*$^{+/+}$. $^{\$}$ $p<0.001$ vs *Trpv5*$^{+/+}$.

Figure 5

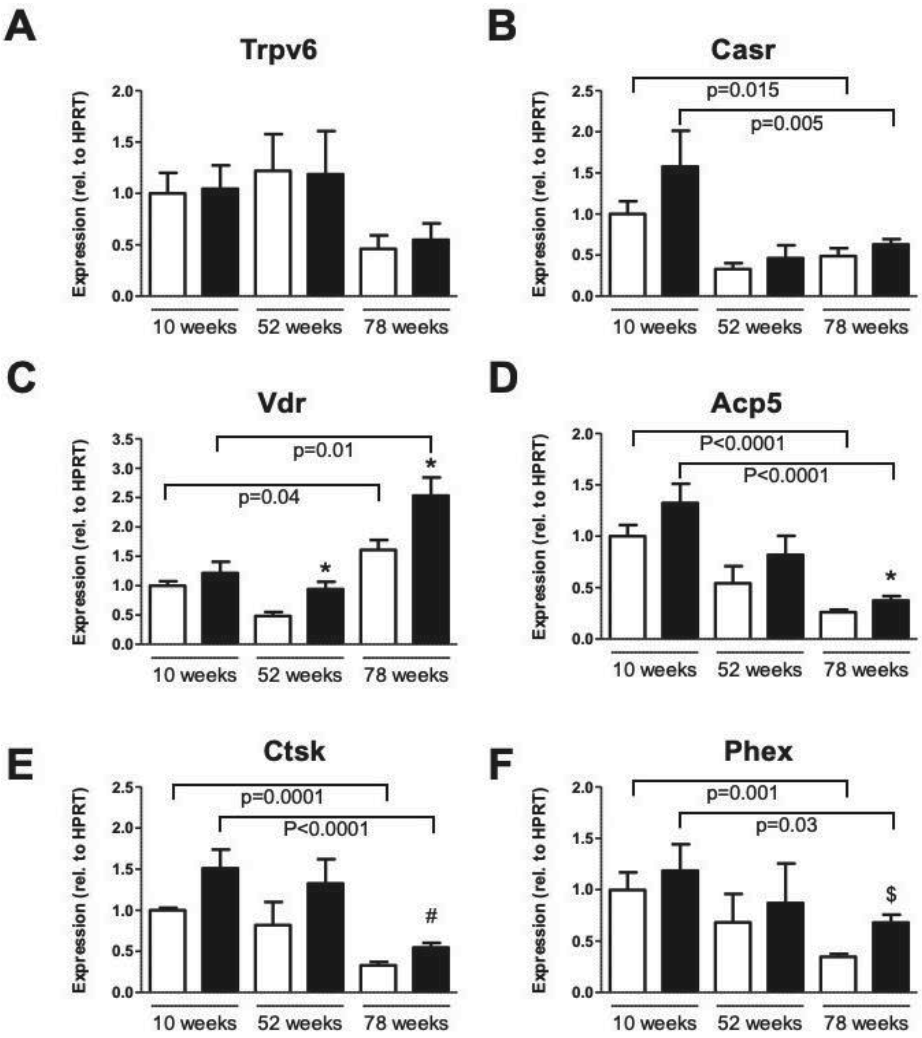

Figure 5: Aging *Trpv5*$^{-/-}$ mice demonstrate high *Vdr* expression
Total femoral RNA was isolated from male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice at 10, 52 and 78 weeks of age (n=3-12). A) *Trpv6*, B) *Casr*, C) *Vdr*, D) *Acp5*, E) *Ctsk* and F) *Phex* mRNA. Gene expression was corrected for the housekeeping gene *Hprt* and expression in *Trpv5*$^{+/+}$ at 10 weeks was set to 1. Normalized for the housekeeping gene Values are presented as mean ± SEM. * p<0.05 versus *Trpv5*$^{+/+}$. $^{\#}$ p<0.01 vs *Trpv5*$^{+/+}$. $^{\$}$ p<0.001 vs *Trpv5*$^{+/+}$. Significant aging effects for a genotype are indicated by horizontal lines.

Discussion

Trpv5 deficiency strongly stresses the Ca^{2+} homeostasis in order to maintain stable plasma Ca^{2+} levels [3, 6]. Here, we demonstrate that mice lacking Trpv5 are able to maintain similar serum Ca^{2+} levels as their *Trpv5*-expressing counterparts during aging up to at least 78 weeks of age. This is

achieved by pronounced age-related increments in serum $1,25(OH)_2D_3$ and PTH. Age-related increases of these hormones were also observed in *Trpv5*$^{-/-}$ mice but interestingly at 10 weeks of age $1,25(OH)_2D_3$ levels were already at the level of older (52 weeks) *Trpv5*$^{+/+}$ mice, pointing to premature aging in this respect in *Trpv5*$^{-/-}$ mice. The stress on the Ca^{2+} homeostasis is paralleled by a reduced degree of bone mineralization and accelerated changes in bone microarchitecture as exemplified by reduced bone thickness.

Normocalcemia in Trpv5$^{-/-}$ mice is associated with elevated serum $1,25(OH)_2D_3$ and PTH

Trpv5$^{-/-}$ mice predominantly suffer from renal calcium loss, which is initially compensated by increased intestinal calcium hyperabsorption through TRPV6 followed by reduced bone mass and mineralization, to retain normocalcemia [3]. This compensatory mechanism is accompanied by elevated levels of $1,25(OH)_2D_3$ and PTH already at 10 weeks of age. Later in life, $1,25(OH)_2D_3$ levels further increase in *Trpv5*$^{+/+}$ mice even to levels exceeding 1300 pmol/l. These extremely high $1,25(OH)_2D_3$ levels may explain that at 78 weeks of age the PTH levels in the *Trpv5*$^{-/-}$ are lower than in the *Trpv5*$^{+/+}$ mice. The continuous exposure of an organism to elevated PTH and $1,25(OH)_2D_3$ levels has adverse consequences for the skeleton. Circulating levels of PTH rise with age in rats and humans [9, 10]. From human clinical studies, it has become apparent that prolonged exposure to high PTH (p.e. primary hyperparathyroidism) indirectly increases osteoclast activity and decreases age-related osteoblast replicative activity [14-17], both contributing to bone loss.

Lieben *et al*. showed that exposure to high $1,25(OH)_2D_3$ levels leads to reduced bone mineralization in mice [18]. In the current study, we demonstrate upregulation of the *Vdr* gene at the level of the femoral bone and strongly elevated $1,25(OH)_2D_3$ levels in the oldest *Trpv5*$^{-/-}$ age group. Although we have no data to support this, upregulation of *Vdr* expression and high serum $1,25(OH)_2D_3$ levels would facilitate enhanced vitamin D signaling within bone. Enhanced vitamin D signaling may thus be a mechanism to limit the influx of Ca^{2+} into bone, which would contribute to retaining serum Ca^{2+} but at the expense of bone mineralization.

Another mechanism that may contribute to retaining normocalcemia is osteocytic osteolysis. Through this process, osteocytes are capable of resorbing the lacunae around them, resulting in the liberation of ions in case of a strong Ca^{2+} demand, such as lactation [19-21]. Suffering from a chronic negative Ca^{2+} balance but keeping serum levels constant, osteocytic osteolysis in

Trpv5$^{-/-}$ mice may suffice to restore serum Ca^{2+}, which in turn would cause the observed increased cortical porosity. Although minute changes in osteocyte function may have great impact due to their numbers within bone, we failed to show detectable changes in osteocytic lacunar size or number at 78 weeks of age.

Mineralization of bone is affected in *Trpv5*$^{-/-}$ mice

In concordance with a chronic Ca^{2+} insufficiency in *Trpv5*$^{-/-}$ mice are our findings on bone mineralization density. Although no effects were seen at the level of the tibial cortex, mineralization density was decreased in the trabecular bone along with non-significantly larger areas of low mineralized bone observed in the 52- and 78-week-old *Trpv5*$^{-/-}$ mice compared to *Trpv5*$^{+/+}$ mice. As we have shown previously, the enhanced intestinal Ca^{2+} uptake could not fully compensate for the renal Ca^{2+} loss in young *Trpv5*$^{-/-}$ mice, leading to reduced skeletal mineralization [6]. Later in life, the persisting stress on Ca^{2+} homeostasis may have led to the observed increased undermineralization in old *Trpv5*$^{-/-}$ mice but also to the increased endocortical osteoclast and osteocyte activity.

Trpv5 deficiency accelerates bone loss

Aging is associated with bone loss by thinning of cortical bone and loss of the trabecular network [22]. Indeed, we found a decrease in cortical thickness with aging and a reduction of the trabecular bone volume fraction and connectivity, despite increased thickness of the remaining trabecular bone. Previous studies looking at bone microarchitecture in aging mice found similar results [23-25]. Glatt *et al* observed an age-related decline in the trabecular bone volume fraction (BV/TV) [23], something we saw until the age of 52 weeks but not thereafter, despite a significant age trend. In line with our data, Ferguson *et al*. described cortical expansion by periosteal apposition in male mice to compensate for trabecular bone loss and reduced mineralization [24]. Hamrick and coworkers showed BMD loss, periosteal expansion and endosteal resorption up to 29 months of age [25]. Interestingly, a concurrent increase in endosteal apposition was observed at this age, for which no explanation was provided but it may be to counteract excessive endosteal bone loss. Nevertheless, it may provide an anabolic time-window for interventions to improve bone quantity/quality later in life. We did not observe differences in bone resorption between 10 and 78 weeks of life as measured by urinary DPD, which corresponds with the study by Hamrick, who showed increased bone resorption only after 18 months of age [25].

Trpv5 deficiency led to a more accelerated bone aging phenotype, including reduced trabecular and cortical bone mass as well as increased endocortical bone volume later in life compared to *Trpv5*$^{+/+}$ mice. For example, the *Trpv5*$^{-/-}$ mice have a cortical thickness at 10 weeks of age that is not reached until *Trpv5*$^{+/+}$ at 78 weeks of age. Despite having a bone resorption defect in early life [6] and reduced urinary DPD levels in *Trpv5*$^{-/-}$ mice at 52 weeks, the current study showed that endocortical bone resorption in *Trpv5*$^{-/-}$ mice exceeds that of *Trpv5*$^{+/+}$ mice between 52 and 78 weeks of age, resulting in an enlargement of the marrow space (endocortical volume) at the level of the diaphysis. In fact, bone resorption was not different between the genotypes, *in vivo* and *ex vivo*, as shown by DPD levels, histomorphometry and bone marrow-derived osteoclast cultures. Apparently, old *Trpv5*$^{-/-}$ mice seem to be able to regain their bone resorption capacity, independent of TRPV5. Perhaps, prolonged high levels of circulating $1,25(OH)_2D_3$, PTH or another yet unknown mechanism following TRPV5 deficiency has a restorative effect at the level of osteoclast activity on the skeleton of these mice. This suggests that the reduced bone resorption caused by TRPV5 deficiency that we reported previously for mice at 10 weeks of age [6] and is apparent by the decreased DPD levels at 52 weeks, may not have been entirely intrinsic and is overcome at older age.

Bone strength is not compromised in old *Trpv5*$^{-/-}$ mice

The combination of reduced bone mass and increased endosteal resorption suggests that life-long stress on calcium homeostasis due to TRPV5 deficiency weakens the skeleton of old mice. Besides the effects on cortical bone mass, we observed increased cortical porosity during aging but especially in the 78-week-old mice lacking TRPV5. Cortical porosity has been appreciated as an age-related phenomenon in the elderly and an important determinant of bone strength but the precise mechanism behind this process remains unclear [26]. Besides these effects on the skeleton of *Trpv5*$^{-/-}$ *mice*, the 3-point-bending tests yielded similar outcomes for both genotypes at 78 weeks of age. The reason for this remains obscure, but may be due to a combination of 1) changes in the composition or the organization of the extracellular matrix leading to structural changes within the skeleton that could not be assessed through 3-point bending; 2) reduced mineralization in *Trpv5*$^{-/-}$ mice that renders bone to be more flexible; 3) non-significantly increased cortical perimeter in *Trpv5*$^{-/-}$ mice between 52 and 78 weeks of age (1.5 mm^3) compared to *Trpv5*$^{+/+}$ mice (1.2 mm^3) that may aid to counteract loss of bone strength. Finally, mice may possess a yet unknown 'compensation' mecha-

nism that in case of bone loss protects them from a fracture, something that is rarely seen in mice.

Human Ca^{2+} homeostasis and bone metabolism

Several disease states, such as chronic kidney diseases and intestinal malabsorption syndromes (e.g. celiac disease, intestinal surgery, aging and vitamin D deficient nutrition) in humans cause continuous stress on the maintenance of adequate serum Ca^{2+} levels, and a clear relationship has been established with bone deterioration [27-30]. The phenotype of the *Trpv5*$^{-/-}$ mice suggests that maintenance of adequate circulatory Ca^{2+} in these patients should be a priority in order to prevent bone loss and increased risk fractures at older age. In conclusion, this study demonstrates that TRPV5 is important for normal bone development and that an unfavorable Ca^{2+} balance persisting during aging leads to accelerated bone loss in *Trpv5*-deficient mice. The strongly elevated vitamin D levels in *Trpv5*$^{-/-}$ mice may aid in restoring serum Ca^{2+} but at the expense of maintaining bone mass and/or mineralization *in vivo*. The TRPV5 deficient mouse appears to be a suitable model for lifelong challenge of calcium homeostasis and its consequences for bone metabolism.

Material and Methods

Animals

Male homozygous TRPV5 null (*Trpv5*$^{-/-}$) and *Trpv5*$^{+/+}$ mice were generated as described before [3]. and were fed standard chow and given water *ad libitum*. At the age of 10, 52 and 78 weeks, mice were sacrificed (group sizes varied between 4-12) and serum, urine and bones were collected. The animal ethics board of the Radboud University Nijmegen approved all animal experimental procedures.

Analytical procedures

Serum Ca^{2+} was calorimetrically determined with a Ca^{2+} assay kit (Sigma) according to the manufacturer's description at 595 nm, using a Bio-Rad microplate reader (Bio-Rad) or using a home-made calcium assay as described before [31]. Serum PTH levels were measured using a mouse intact PTH ELISA kit following standard procedure (Immutopics, San Clemente, CA, USA). Serum $1,25(OH)_2D_3$ concentrations were measured by immunoextraction followed by quantitation by ^{125}I-RIA (IDS, Boldon, UK) [6]. Urinary total deoxypyridinoline (DPD) cross-links were determined, using the Metra DPD assay according to the guidelines (Quidel).

Microcomputed tomography (µCT)

Femurs were scanned using the SkyScan 1072 microtomograph (Bruker MicroCT) with a resolution of 7 µm and subsequently analyzed as described in detail before [6]. According to guidelines recently published [32] the following settings were used: X-Ray power and tube current were 40 kV and 0.25 mA, respectively. Beam hardening (20%) was reduced using a 1 mm aluminum filter, ring-artefacts were reduced (set at 5), exposure time was 5.9 seconds and an average of three pictures was taken at each angle (0.9º) to generate final images. Using different software packages from Bruker MicroCT (NRecon, CtAn and Dataviewer), bone microarchitectural parameters were assessed in trabecular and cortical bone of all mice (n=14 for both genotypes). The trabecular bone parameters trabecular tissue volume, bone volume, trabecular volume fraction (BV/TV), trabecular thickness, trabecular number, connectivity density and structure model index were determined in the distal metaphysis of the femur (scan area 0 – 3.25 mm of proximal femur). In the mid-diaphysis (scan area 3.25 - 6.3 mm from trochanter), cortical volume, cortical thickness, cortical porosity, polar moment of inertia (MOI; proxy for bone strength) and perimeter were analyzed. For image processing, trabecular bone was manually selected and cortical bone was automatically selected. We used global thresholding for segmentation, followed by applying optimized threshold levels for trabecular and cortical bone measurements.

Bone mechanical properties (3-point bending)

Femurs were stored in phosphate-buffered saline at -20°C until further use. Before the 3-point bending test, femurs were scanned according to the settings mentioned above. The procedure was carried out as previously described in detail [33]. Briefly, femurs were placed in a custom made 3-point bending device, with the lower loading posts 10 mm apart. Mechanical testing was performed, using a Single Column Lloyd LRX System (Lloyd Instruments). Displacement (mm) and force (N) were registered. Using the same settings for filtration, segmentation and binarization as mentioned above in the µCT section, the MOI, reflecting the ability of the bone to withstand torsion, was calculated using Ct Analyzer software (Bruker MicroCT). This was determined in the µCT scan-derived cross-section that corresponded to the fracture site resulting from the bending test. From the resulting displacement to force graphs as well as the MOI values, ultimate force (N), stiffness (N/mm), energy (Nmm) and elastic modulus (MPa) were determined as described before [34].

Quantitative backscattered electron imaging

Dissected tibiae were routinely fixed and dehydrated in ethanol, and embedded in polymethylmethacrylate. Sample blocks were trimmed using a low speed diamond saw (Isomet-R, Buehler Ltd.). Sectioned bone surfaces were sequentially ground with sand paper with increasing grid number followed by polishing with diamond grains (size down to 1 micron) on hard polishing clothes by a precision polishing device (PM5 Logitech, Glasgow, Scotland). Finally the sample surface was carbon coated by vacuum evaporation (Agar SEM Carbon Coater) for scanning electron microscopy.

Bone mineralization density distribution (BMDD) was determined in the metaphyseal spongiosa and in the midshaft cortical bone by qBEI using a digital scanning electron microscope (DSM 962, Zeiss) equipped with a four-quadrant semiconductor backscattered electron detector as extensively described before [35, 36]. The accelerating voltage of the electron beam was set to 20 kV, the probe current to 110 pA, and the working distance to 15 mm. The cancellous and cortical bone areas were imaged at 200x nominal magnification (corresponding to a pixel resolution of 1 µm/pixel. From these digital images, grey level histograms were deduced, displaying the percentage of bone area occupied by pixels of a certain gray level. The transformation of these into calcium weight percent (wt%Ca) histograms led to a bin width of 0.17 wt%Ca. A technical precision of 0.3% was achieved. The BMDD parameters like the mean (weighted mean) CaMean and most frequently occurring calcium concentration CaPeak (peak position of the BMDD) in the sample, the width of the distribution CaWidth (full width at half maximum; Δwt%Ca) reflecting the heterogeneity in matrix mineralization and CaLow, the percentage of bone below 17.68 wt%Ca (primary mineralization)[37].

Osteocyte density measurements

qBEI Images of cortical bone (nominal magnification 200X) with 1 □**m** pixel resolution were used to determine osteocyte lacunae number. The mean size was evaluated from the 2D images of the lacunae as well as the percentage of the sectioned bone surface occupied by these lacunae (density). This was done, using the software package Bioquant (Version 7.20; Bioquant image analysis corporation).

Bone histomorphometry

After excision, femurs were routinely embedded in methylmetacrylate as described before [6]. Six µm sections were subjected to tartrate-resistant acid phosphatase (TRAP) staining. Sections were deacrylated, hydrated and

rinsed in 0.2 M sodium acetate / 50 mM tartaric acid for 5 minutes. Naphtol AS-MX (0.5 mg/ml) and 1.1 mg/ml Fast red TR salt (both from Sigma) were added and incubated for 120 minutes at 37°C. Counterstaining was performed with haematoxylin for 5 seconds and after air-drying, the sections were embedded in Permount (Thermo Fischer Scientific). Images were taken with a Nikon Eclipse E400 system (Nikon, Lijnden, the Netherlands) and osteoclast number (Oc.N) and surface (Oc.S) were determined per total bone surface (BS), using Bioquant software.

Bone marrow cultures

Bone marrow cells derived from 78-week-old mice stimulated towards osteoclasts and osteoblasts were cultured as described in detail [6, 38]. After 6 days of osteoclast culture, TRAP and coomassie brilliant blue stainings were used to stain for osteoclasts and resorption pits on bone slices left behind by osteoclasts, respectively [6]. Osteoclast number and resorption surface were determined and resorption surface per osteoclast was calculated, using the freely available ImageJ software (version 1.41; http://rsbweb.nih.gov/ij/). Osteoblast at day 14 cells were washed with PBS and taken up in TriZol for isolation of total RNA as described below.

Flowcytometric analysis

Frequencies of bone marrow populations containing osteoclast precursors were determined by flowcytometry, essentially as described in de Vries *et al*. [13]. Bone marrow cells from approximately 10- and 22-month-old mice were collected, washed and counted. All immunofluorescent labelings and washes took place in FACS buffer (PBS containing 1% BSA). Bone marrow cell suspensions were spun down at 1500 rpm for 5 min at 4ºC) and incubated for 30 min in 25 µl per 10^6 cells biotinylated ER-MP12, recognizing CD31 [39]. Cells were washed once and incubated in 25 µl per 10^6 cells FACS-buffer containing FITC–conjugated ER-MP20, recognizing Ly6C [39], and streptavidine-phycoerythrin (PE; Becton Dickinson; 10 µl per 10^6cells) for 30 min. Finally, cells were fixed in 1% vol/vol paraformaldehyde in PBS for 15 min, washed and taken up in PBS. Cells were analyzed using a BD FACS Calibur and data were processed using Diva software (Becton Dickinson).

RNA isolation, cDNA synthesis and real-time PCR

Pulverized material from mouse femurs (including bone marrow) was resuspended in Trizol Reagent (Gibco). RNA isolation, cDNA synthesis and real-time PCR on these femur samples as well as on the samples from the bone

marrow cultures were performed as described previously [40]. Primer and probe sequences and concentrations used for real-time PCR are listed in Supplementary table 1. The gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as an internal control to normalize for differences in RNA extraction and degradation as well as for efficiency of the cDNA synthesis. Data were presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ (ΔCt (cycle threshold) = Ct of gene of interest minus Ct of housekeeping gene).

2.11 Statistics

In all experiments values are expressed as mean ± SEM unless stated otherwise. Differences between genotypes were tested for significance by AN(C)OVA and adjusted for age. In the *in vivo* studies, regression analyses were performed to assess age-related changes. Two-way ANOVA was performed to assess for interaction between age and genotype. Values were considered significantly different at $p < 0.05$.

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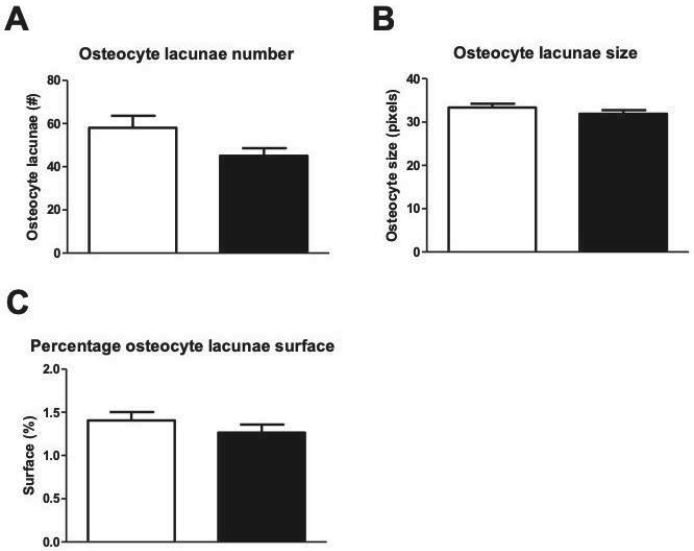
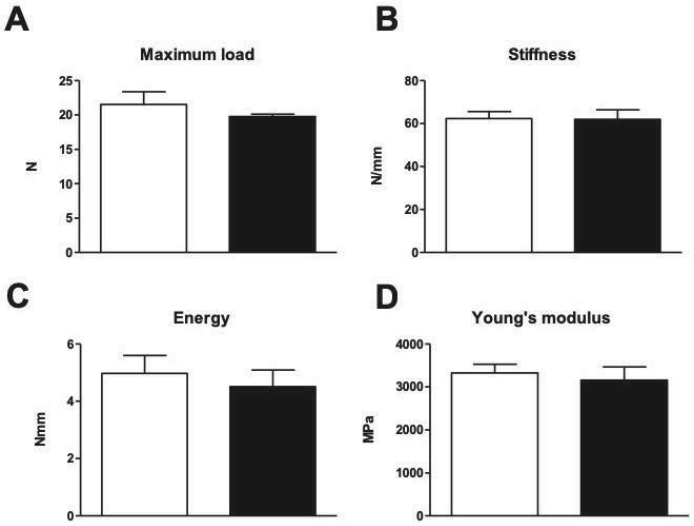
Supplemental figures

Supplementary figure 1



Supplementary figure 1: Osteocyte lacunae are not affected in old *Trpv5*$^{-/-}$ mice
Femoral bone sections of male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice were used to determine A) osteocyte lacunae number, B) osteocyte lacunae size and C) percentage osteocyte lacunae surface/bone surface. Values are presented as mean ± SEM.

Supplementary figure 2



Supplementary figure 2: Bone strength is not affected in old *Trpv5*$^{-/-}$ mice
Femurs of male *Trpv5*$^{+/+}$ (white bars) and *Trpv5*$^{-/-}$ (black bars) mice were subjected to 3-point-bending (n=6-9). A) Maximum load, B) Stiffness, C) Energy and D) Young's modulus were measured. Values are presented as mean ± SEM.

Supplemental tables

Supplementary table 1: Murine primer sequences for SYBR-green-based Q-PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Quantity (pmol)
Hprt	TTATCAGACTGAAGAGCTACTGTAATGATC	TTACCAGTGTCAATTATATCTTCAACAATC	2.5
Trpv6	TTCCAGCAACAAGATGGCCTCTACTCTGA	ATCCGCCGCTATGCACA	10
S100g	CCTGCAGAAATGAAGAGCATTTT	CTCCATCGCCATTCTTATCCA	10
Ncx1	TCCCTACAAAACTATTGAAGGCACA	TTTCTCATACTCCTCGTCATCGATT	10
Atp2b1	CGCCATCTTCTGCACCATT	CAGCCATTGCTCTATTGAAAGTTC	10
Ctsk	TGATGAAAATTGTGACCGTGATAA	CTCTCTCCCCAGCTGTTTTTAATTA	2.5
Clcn7	CCTGTGGTGGAGGATGTAGGA	TCTCCACAAACACCTTATGCTT	5
Acp5	AAGAACTTGCGACCATTGTTAGC	CCTGAAGATACTGCAGGTTGTGG	2.5
Tcirg1	TCAGATCCTAAGCCGAAGTTGAG	GACCAAGGCCACCTCTTCAC	5
Vdr	GATCTTGTCAGTTACAGCATCCAAAA	CGCAACATGATCACCTCAATG	2.5
Casr	CTTTCCTATCCATTTTGGAGTAGCA	GCAAAGATCATGGCTTGTAACCA	10
Ctr	GTGATGAAAATGGAGAGTGGTTTAGA	AAGAACGTACGCATTTTGCAGT	2.5
Kl	CCAAAAGCTGATAGAGGACAATGG	GTGTCCACTTGAACGTAGTTGTCAA	5
Sost	ACCTCCCCACCATCCCTATG	TGTCAGGAAGCGGGTGTAGTG	2.5
Ankh	TACAGAGGCAGTGGCCATTCT	GTTGCTTGTGTTCGCCAGTTT	5
Spp1	AAGGATGACTTTAAGCAAGAAACTCTTC	TCCTCGCTCTCTGCATGGT	2.5
Fgf23	CATCTACAGTGCCCTGATGATTACA	CTTCGAGTCATGGCTCCTGTT	5
Phex	AACCGAACCAGTGAGGCTATGT	TAGAGTCTGGTATCAATGACCTTCTTG	2.5

Supplementary table 2: Comparison of serum calcium levels in *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice during aging

	10 weeks	52 weeks	WT mice previous cohort
	Trpv5$^{+/+}$	*Trpv5*$^{+/+}$	WT
	mean ± sem	*mean ± sem*	*mean ± sem*
Serum			
Calcium (mmol/l)	2.76 ± 0.02	2.71 ± 0.04	2.56 ± 0.04

Supplementary table 3: µCT data of femurs from male *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice during aging

	10 weeks		52 weeks		78 weeks	
	Trpv5$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$
	mean ± sem	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*
Metaphysis						
Trabecular thickness (µm)	69.5 ± 1.5	68.0 ± 1.6	75.2 ± 2.3	68.1 ± 0.8 [a]	80.3 ± 2.5 [d]	72.5 ± 1.5 [a,e]
Trabecular volume (mm^3)	1.5 ± 0.1	1.3 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Trabecular bone volume fraction (BV/TV; %)	30.6 ± 1.7	26.4 ± 1.5	16.6 ± 1.1	18.4 ± 2.5	20.6 ± 1.4	19.5 ± 2.0
Trabecular number (mm^{-1})	0.73 ± 0.02	0.75 ± 0.02	0.41 ± 0.04	0.53 ± 0.06 [a]	0.46 ± 0.05 [d]	0.50 ± 0.04 [e]
Trabecular separation (µm)	271 ± 8	285 ± 9	383 ± 16	380 ± 45	407 ± 21 [d]	391 ± 26 [e]
Connectivity density (mm^{-1})	449 ± 59	317 ± 17	51 ± 16	121 ± 43	89 ± 24 [d]	122 ± 33 [e]
Structure model index	1.6 ± 0.1	1.7 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.1 [d]	2.0 ± 0.1 [e]
Diaphysis						
Cortical volume (mm^3)	4.0 ± 0.2	3.4 ± 0.2 [a]	4.1 ± 0.1	3.7 ± 0.1 [a]	4.1 ± 0.2	3.6 ± 0.1 [a]
Cortical thickness (µm)	275 ± 6	238 ± 7	256 ± 8	224 ± 2	225 ± 10 [d]	195 ± 10 [e]
Endocortical volume (mm^3)	2.7 ± 0.2	2.8 ± 0.2	4.3 ± 0.2	4.4 ± 0.1	4.6 ± 0.2 [d]	5.9 ± 0.3 [e]
Cortical porosity (% of Ct.V)	0.22 ± 0.06	0.16 ± 0.04	0.25 ± 0.08	0.32 ± 0.06	0.56 ± 0.06 [d]	0.93 ± 0.14 [a,e]
Polar moment of inertia (mm^4)	0.68 ± 0.07	0.54 ± 0.06	0.90 ± 0.05	0.83 ± 0.05	1.02 ± 0.05 [d]	1.04 ± 0.05 [e]
Perimeter (mm)	5.6 ± 0.1	5.32 ± 0.15	6.4 ± 0.3	6.3 ± 0.4	7.6 ± 0.1 [d]	7.8 ± 0.2 [e]

[a] $p<0.05$ versus *Trpv5*$^{+/+}$ mice of the same age, [b] $p<0.01$ versus *Trpv5*$^{+/+}$ mice of the same age, [c] $p<0.001$ versus *Trpv5*$^{+/+}$ mice of the same age. [d] $P<0.05$ for age trend in *Trpv5*$^{+/+}$ mice. [e] $p<0.05$ for age trend in *Trpv5*$^{-/-}$ mice.

Supplementary table 4: qBEI data of cortical bone from male *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice during aging

	10 weeks		52 weeks		78 weeks	
	Trpv5$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$
	mean ± sem	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*
Diaphyseal cortex						
CaMean (wt% Ca)	22.8 ± 0.3	23.3 ± 0.4	25.6 ± 0.2	24.5 ± 0.3	26.1 ± 0.6 [a]	26.6 ± 0.2 [b]
CaPeak (wt% Ca)	23.7 ± 0.3	24.1 ± 0.4	26.5 ± 0.2	25.5 ± 0.3	27.0 ± 0.6 [a]	27.4 ± 0.3 [b]
CaWidth (Δwt% Ca)	3.12 ± 0.09	3.02 ± 0.09	2.98 ± 0.17	3.06 ± 0.12	4.39 ± 0.35 [a]	3.70 ± 0.21 [b]
CaLow (% bone area)	4.49 ± 0.45	3.76 ± 0.33	3.17 ± 0.11	3.51 ± 0.39	2.32 ± 0.42 [a]	2.38 ± 0.37 [b]

[a] p<0.05 for age trend in *Trpv5*$^{+/+}$ mice. [b] p<0.05 for age trend in *Trpv5*$^{-/-}$ mice.

Supplementary table 5: Mouse bone marrow composition analyzed by flowcytometry in *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice[1]

		10 months		22 months	
	phenotype	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$
Cell population (%)		*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*
Early blasts	CD31^{hi} Ly-6C^{neg}	1.5 ± 0.2	1.6 ± 0.0	1.3 ± 0.3	1.3 ± 0.1
Lymphoid cells	CD31^{int} Ly-6C^{neg}	17.3 ± 1.9	17.4 ± 2.8	19.5 ± 1.5	10.7 ± 1.2 [a]
Erythroid precursors	CD31^{neg} Ly-6C^{neg}	16.8 ± 1.8	18.9 ± 2.0	14.2 ± 0.9	15.3 ± 1.8
Myeloid blasts	CD31^{pos} Ly-6C^{pos}	7.8 ± 0.2	6.5 ± 0.8	8.2 ± 0.2	8.9 ± 0.8
Neutrophils	CD31^{neg} Ly-6C^{int}	22.6 ± 1.4	23.4 ± 1.8	24.1 ± 1.0	29.2 ± 2.4
Monocytes	CD31^{neg} Ly-6C^{hi}	5.6 ± 0.6	3.5 ± 1.0	4.6 ± 0.5	5.5 ± 0.3

[a] p<0.05 versus *Trpv5*$^{+/+}$ mice of the same age
[1] Bone marrow populations were identified as described in de Vries et al. 2009

Supplementary table 6: Gene expression in femurs from *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ mice during aging

	10 weeks		52 weeks		78 weeks	
	Trpv5$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$	*Trpv5*$^{+/+}$	*Trpv5*$^{-/-}$
	mean ± sem	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*	*mean ± sem*
Calcium homeostasis						
Calbindin-D_{9K} (*S100g*)	1.0 ± 0.1	1.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.2 [c]
Sodium/calcium exchanger 1 (*Ncx1*)	1.0 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	1.4 ± 0.1 [a]
Plasma membrane calcium ATPase 1 (*ATP2b1*)	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	1.4 ± 0.3	0.9 ± 0.1	1.3 ± 0.2
Osteoclast markers						
Calcitonin receptor (*Ctr*)	1.0 ± 0.2	1.1 ± 0.2	0.8 ± 0.3	1.3 ± 0.3	0.4 ± 0.1 [b]	0.5 ± 0.1 [c]
Chloride channel 7 (*Clcn7*)	1.0 ± 0.1	1.3 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
H^+-ATPase (*Tcirg1*)	1.0 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
Aging of bone						
Osteopontin (*Spp1*)	1.0 ± 0.0	1.1 ± 0.0	0.8 ± 0.2	0.9 ± 0.2	0.5 ± 0.1 [b]	0.7 ± 0.1 [c]
Sclerostin (*Sost*)	1.0 ± 0.2	1.6 ± 0.4	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0 [b]	0.7 ± 0.1 [c]
Ankylosis, progressive homolog (*Ank*)	1.0 ± 0.2	1.0 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.0
Fibroblast growth actor 23 (*Fgf23*)	1.0 ± 0.3	1.5 ± 0.4	3.1 ± 0.7	1.4 ± 0.3	1.7 ± 0.3	1.8 ± 0.5
Klotho (*Kl*)	1.0 ± 0.3	0.7 ± 0.1	1.4 ± 0.3	0.8 ± 0.3	1.1 ± 0.3	1.0 ± 0.3

Data were normalized to *Trpv5*$^{+/+}$ mice at 10 weeks of age (set to 1.0). [a] $p<0.05$ vs *Trpv5*$^{+/+}$ mice of same age. [b] $p<0.05$ for age trend in *Trpv5*$^{+/+}$ mice. [c] $p<0.05$ for age trend in *Trpv5*$^{-/-}$ mice.

Supplementary table 7: Gene expression in *Trpv5*$^{+/+}$ and *Trpv5*$^{-/-}$ bone marrow-derived osteoblasts and osteoclasts

	78 weeks	
	Trpv5$^{+/+}$	*Trpv5*$^{-/-}$
	mean ± sem	*mean ± sem*
Osteoblast genes		
Collagen 1a1 (*Col1a1*)	108.2 ± 43.9	61.1 ± 9.7
Osteocalcin (*Bglap*)	0.58 ± 0.31	0.22 ± 0.05
Runt-related transcription factor 2 (*Runx2*)	0.48 ± 0.19	0.28 ± 0.02
Osteoclast genes		
Acid phosphatase 5, tartrate resistant *(Acp5*)	39.1 ± 10.7	34.1 ± 7.2
Chloride channel, voltage-sensitive 7 (*Clcn7*)	0.32 ± 0.08	0.28 ± 0.04
T-Cell, Immune Regulator 1, ATPase, H+ Transporting, Lysosomal V0 Subunit A3 (*Tcirg1*)	1.53 ± 0.39	1.22 ± 0.29

Chapter 9

General discussion

General discussion

In this thesis, studies are presented on sex- and age-related challenges in calcium and phosphate homeostasis.

First of all, the findings related to the effects of aging on calcium and phosphate homeostasis from the different studies presented in this thesis will be integrated in the following chapter. Secondly, sex-specific results with respect to calcium and phosphate homeostasis are discussed. The chapter will close with a paragraph on future perspectives based on the findings presented in this thesis.

1. Aging and its relationship with calcium and phosphate homeostasis

The average lifespan has steadily increased since the start of the nineteenth century [1]. Although aging is a natural phenomenon, to date senectitude is still associated with increasing prevalence of chronic illnesses such as cardiovascular diseases, chronic kidney diseases and metabolic diseases like type 2 diabetes and osteoporosis [2-4]. Despite the population getting older, the age when these illnesses manifest has not increased much and in some cases, such as in patients suffering from type 2 diabetes, the age of first manifestation has even gone down [5]. Hence, people live longer but with a reduced quality of life due to impaired health. Health is considered to be a concept described as a continuum between health and a disease state and reflected as a dynamic balance between the demands and the self-management of people. The presence of chronic diseases can put an extra demand on people and potentially reduce self-management [6]. Preventing or postponing the occurrence of chronic diseases with aging can therefore aid the individual's perceived health. At a conference for international health experts in The Hague, The Netherlands in 2009, one of the discussed areas related to health was the ability to maintain homeostasis despite changing circumstances [7, 8]. In order to address healthy aging it is therefore important to understand possible age-related alterations in homeostasis and factors that influence said homeostasis. Serum calcium and phosphate are part of a tightly regulated homeostatic system and both have an important role in many physiological processes. Both serum calcium and phosphate are necessary for the adequate mineralization of bone and are incorporated in the bone matrix as part of hydroxapatite [9]. Furthermore, calcium is necessary for many metabolic processes such as neural transmission, blood coagulation, and cell proliferation, whereas phosphate is both a part of DNA as well as RNA structures and

an important factor in energy metabolism. Age-related alterations in calcium and phosphate homeostasis are examined in this thesis, as they may affect health in various ways.

In chapter 2 we found both sex differences and an age-related decline in serum calcium and phosphate levels. Women above 45 years of age had higher serum calcium and phosphate levels compared to men of a similar age. This sex difference in serum calcium and phosphate levels was not explained by vitamin D or ALP levels. We showed that serum testosterone influenced the sex differences in serum phosphate, but not calcium. Estradiol did not affect sex differences in either serum calcium or phosphate. In chapter 3 we discovered that these sex differences in calcium and phosphate levels became apparent with aging. In three different hospital cohorts, with subjects aged 1-97 years, a sex-dependent difference became apparent, with women having higher serum calcium and phosphate levels than men above the age of 45, but not in younger age groups. Analyses per decade showed that sex differences in serum phosphate appeared roughly 10 years before sex differences in serum calcium were observed. Overall, there were associations related to both sex and age with serum calcium and phosphate throughout the population. In chapter 4 the influence of higher phosphate levels on the risk of fractures and bone mineral density (BMD) in community-dwelling elderly people is described. Higher serum phosphate levels, even within the normal range, were associated with increased fracture risk in both men and women, and decreased lumbar spine BMD in men. The increased fracture risk of the participants in this study could not be explained by BMD. For serum calcium levels no association with fracture risk and BMD was found in either sex (chapter 4).

Calcium homeostasis is maintained by an intricate endocrine system involving multiple organs and hormones. Calcium intake attributes to a small extent to calcium homeostasis, as calcium intake does not equal intestinal absorption. Nevertheless, adequate calcium intake and adequate vitamin D levels are considered important for optimal treatment of osteoporosis [10]. Vitamin D facilitates calcium uptake in the intestines, and is therefore one of the hormones that is important in the maintenance of adequate serum calcium levels.

We wanted to address intestinal calcium handling in relation to bone parameters and calcium levels. In order to study the influence of calcium intake on bone we assessed the influence of lactose intolerance in elderly subjects. Lactose intolerance is associated with reduced calcium intake and in some stud-

ies an association with bone parameters has been found [11-13]. In chapter 5 we describe that lactose intolerance, genetically defined by a polymorphism in the *LPH* gene, is associated with lower calcium intake and lower serum levels of calcium, but not with BMD and fracture risk, suggesting that serum calcium levels within the normal range are adequate for good bone health. A recent genome wide association study and a two-sample mendelian randomisation study confirmed that there was no increased fracture risk in relation to lower vitamin D levels as well as lower estimated calcium intake from dairy sources [14]. Fractures at older age are considered to be one of the major health burdens, since they are associated with both morbidity and mortality [7, 15-17]. People previously living independently might need to move to a nursing home, suffer from pain and have significantly declining physical conditions by decreased mobility [17, 18]. Therefore, it is important to study those aspects of deteriorating bone health with aging, including adequate calcium and phosphate homeostasis, which can lead to more insight into the prevention of fractures. A meta-analysis in 2015 from Bolland et al. found no association between use of calcium supplements and fracture risk [19]. In a subsequent meta-analysis, an influence of calcium intake and supplementation on BMD was found, although this was considered to be marginal with an increase in BMD of 1-2% [20]. Extrapolation of previous epidemiological studies was used to estimate that this increase of 1-2% in BMD would result in a fracture risk reduction of 5-10% [20]. However, the aforementioned researchers could not find a reduced fracture risk with the use of calcium supplements in their previous meta-analysis [19]. In a Cochrane systematic review by Avenell et al., associations were found between calcium and vitamin D supplementation and decreased fracture risk, but not for vitamin D alone [21]. This implicates that supplementation of either calcium or vitamin D alone is insufficient to reduce fracture risk. However, calcium and vitamin D supplementation together seems to be a beneficial combination when trying to reduce fracture risk. Moreover, it has been shown that patients with conditions that affect intestinal calcium uptake and vitamin D absorption, such as celiac disease and short bowel syndrome, have lower BMD and increased fracture risk [22, 23]. Calcium and vitamin D supplements are therefore recommended in order to prevent further bone loss [24-26].

Low levels of serum calcium and phosphate levels can result in severe bone phenotypes such as rickets in children and osteomalacia in adults due to inadequate bone mineralization in, for example, patients with chronic kidney disease, and in severe vitamin D deficiency or in hypophosphatemic rickets.

We describe a case of pseudovitamin D-deficient rickets in chapter 6. These diseases illustrate the importance of an adequate calcium and phosphate homeostasis for the healthy development of bone. To date, no reference values for serum calcium and phosphate are established for people > 60 years of age and abnormalities in serum calcium and phosphate levels in people > 60 years of age are based on the reference values of younger people [27, 28]. Misinterpretation of the adequacy of serum values, such as serum calcium and phosphate, can occur when the possibility of alterations in homeostasis with healthy aging is ignored. Therefore, a Swiss study group started in 2011 recruiting people aged > 60 older in the SENIORLAB study, in order to set reference values for different markers of metabolism and electrolytes for healthy elderly people [29]. Presently, these reference values are not available, but in the future, these reference values might help in addressing abnormalities in serum calcium and phosphate homeostasis in people >60 years of age.

One of the gatekeepers in renal calcium handling is transient receptor potential (TRP) cation channel subfamily V, member 5 (TRPV5). TRPV5 is a calcium channel that is present in the distal part of the nephron in the kidney, where it is involved in the reabsorption of calcium [30]. Moreover, TRPV5 is present in osteoclasts, where it plays a role in adequate osteoclastic bone resorption [31]. In chapter 8 we describe an aging study in mice in which the maintenance of adequate calcium levels was challenged as a consequence of a defective calcium channel TRPV5. Lacking this calcium channel disturbs the reabsorption of calcium in the kidneys and impairs osteoclastogenic bone resorption. During aging, *Trpv5* null mice developed accelerated bone loss and a diminished bone mineralization phenotype. A previous study involving similar mice showed that in both *Trpv5* null mice and wild types, aging increased urinary calcium excretion. However, *Trpv5* null mice had significantly higher urinary calcium excretion compared to wild types [32]. Intriguingly, we found that *Trpv5* null mice had increased levels of Vitamin D and parathyroid hormone (PTH) at an earlier age compared to control mice, while serum calcium levels were not different between the different genotypes. The increased Vitamin D and PTH levels, together with the previously found increased urinary calcium excretion, in these *Trpv5* null mice, while serum calcium levels remain normal, implicates that the body attempts to maintain serum calcium levels. We concluded that serum changes in vitamin D and PTH may be responsible for the observed accelerated bone loss in mice lacking *Trpv5*. Previous studies using mice without a functional Vitamin D re-

cepetor, *Vdr* null mice, and mice with a deletion of the 25-hydroxyvitamin D_3 1a-hydroxylase enzyme, the so called *Cyp27b1* null mice, have shown deleterious effects of these mutations on bone mineralization. A rescue diet rich in calcium, phosphorus and lactose resulted in normalization of calcium, phosphorus and PTH levels, but not always in complete normalization of the bone phenotype. This indicates the importance of vitamin D in bone mineralization [33, 34]. It has to be mentioned that compared to humans, vitamin D affects bone of mice differently. In both species the most potent form of vitamin D, 1,25-dihydroxyvitamin D_3, is synthesized by hydroxylation of 25-hydroxyvitamin D_3 via the CYP27B1 enzyme in the kidney. In humans, the main effect of 1,25-dihydroxyvitamin D_3 on bone is the ability of 1,25-dihydroxyvitamin D_3 to stimulate the intestinal uptake of calcium and phosphate, thereby providing sufficient minerals available for incorporation in bone [35]. In contrast to humans, 1,25-dihydroxyvitamin D_3 can have both anabolic as well as catabolic effects on bone in mice [33]. In mice, the direct anabolic effect of 1,25-dihydroxyvitamin D_3 occurs through activation of the VDR receptor in mature osteoblast, which leads to a reduced RANKL-OPG ratio, inhibiting osteoclast maturation and activity. Furthermore, 1,25-dihydroxyvitamin D_3 stimulates the LRP5 pathway, leading to bone formation. Indirectly, in mice 1,25-dihydroxyvitamin D_3 exerts its anabolic effects via intestinal calcium and phosphate uptake in order to facilitate sufficient minerals for bone mineralization. The catabolic action of 1,25-dihydroxyvitamin D_3 on bone in mice also has a direct and an indirect effect. The direct catabolic effect of 1,25-dihydroxyvitamin D_3 in mice affects bone through the VDR receptor in early osteoblasts, which increases the RANKL-OPG ratio under the influence of 1,25-dihydroxyvitamin D_3, resulting in increased osteoclastogenesis and, consequently, in bone resorption. In mature osteoblast and osteocytes in mice, 1,25-dihydroxyvitamin D_3 can lead to increased production of local inhibitors of bone mineralization such as ENPP1, ENPP2 and ANK [33]. Furthermore, in mice, 1,25-dihydroxyvitamin D_3 increases FGF23 levels, resulting in lower serum phosphate levels, as a consequence of which less phosphate is available for incorporation in the bone matrix [33]. Because the different actions of 1,25-dihdroxyvitamin D_3 in mice are both catabolic and anabolic, whereas 1,25-dihydroxyvitamin D_3 is only anabolic on human bone,

the results in chapter 8 are not directly translational to humans. Nevertheless, the importance of the TRPV5 channel in maintaining adequate calcium balance in mice has provided insight into the molecular mechanism of calci-

um homeostasis in aging; that is, when calcium homeostasis is put under stress.

In 2015, a nonsynonymous SNP (rs4236480) in the *TRPV5* gene was found to be associated with nephrolithiasis [36], but to date no case reports on *TRPV5* gene malfunctioning in humans have been published. Despite the clear function of the TRPV5 calcium channel in the epithelial cells in the kidney, as well as in osteoclasts, overall malfunctioning of the *TRPV5* gene might only lead to a clinical phenotype in conditions when calcium homeostasis is put under severe stress. It could therefore be that TRPV5 in humans, over the course of life, has an indirect impact on bone health when the maintenance of calcium homeostasis is challenged due to decreased intestinal calcium uptake, decreased renal function and/or lower levels of vitamin D.

Besides osteoporosis, our findings on disturbances of calcium and phosphate homeostasis in this thesis may also be relevant for cardiovascular diseases such as coronary artery disease, another chronic age-related condition which is associated with increased extra-skeletal calcification. Foley and coworkers found serum calcium, serum phosphate and the calcium-phosphate product to be associated with cardiovascular disease and stroke [37]. Osteoporosis and atherosclerosis have been linked as reviewed by Anagnostis et al. [38]. Two distinct molecular pathways that are activated with aging are considered to play an important role in osteoporosis as well as atherosclerosis [39]. The first pathway is through inflammation, resulting in cytokine release, which leads to increased bone resorption and diminished bone formation in the bone microenvironment. In vascular tissue the same molecular pathway of cytokine release causes vascular smooth muscle cells to adopt a more osteogenic phenotype, partly via the activation of the Wnt-Beta catenin signaling cascade, causing calcification of the vascular tissue [40]. The second molecular pathway, activated later in life in bone and vascular tissues, is a pathway that is stimulated by oxidative stress. In bone, the increased product of lipid oxidation due to oxidative stress leads to increased osteoclast differentiation and decreased osteoblast formation [41]. In vascular smooth muscle cells oxidative stress has been shown to cause lipid oxidation *in vitro*, leading to increased calcification of these vascular smooth muscle cells [41].

Overall, it is clear that understanding the molecular pathways behind calcium and phosphate homeostasis with aging is crucial not only for bone, but also for other tissues and processes. It helps to understand how changes in serum calcium and phosphate levels can be related to certain conditions such as osteoporosis and cardiovascular diseases. Unraveling the common denom-

inators of the molecular basis in diseases such as osteoporosis and atherosclerosis may yield new targets for therapeutic interventions influencing both conditions.

1.2 Biomarkers and aging

With advancing age there is an increase in osteoclastic bone resorption, causing calcium and phosphate to be released from the bone matrix into the circulation [42, 43]. When osteoclastic bone resorption is not matched with bone formation, BMD decreases and fracture risk increases [44]. In chapter 7 we studied the osteoclastogenic potential of peripheral mononuclear cells in elderly women with and without osteoporosis. We found an association between deoxypyridinoline (DPD) levels, a marker of bone resorption, and osteoclastogenic potential in women diagnosed with osteoporosis, but not in women without osteoporosis. There was a difference in osteoclastogenic potential in osteoporotic subjects stratified according to high or low DPD levels. Osteoporotic subjects with high DPD levels had a higher osteoclastogenic potential compared to osteoporotic subjects with low DPD levels. It appears that osteoporotic subjects can be divided in two distinct metabolic groups: a group with high and a group with low DPD levels. Considering the finding that osteoclastogenic bone resorption is associated with DPD levels, we speculate that there might be a difference in fracture risk in these metabolically distinct groups. Notable is that in our study in chapter 7 the overall DPD levels were low, whereas in previous studies high bone resorption markers were found in women shortly after menopause [45-48]. Since the women in our study were on average 78 years of age, we may consider that the body's metabolism is low due to older age, and that bone frailty in the elderly osteoporotic subjects with low DPD levels in chapter 7 may be more associated with an overall decreased formation and a stable, but not increased, bone resorption. This phenotype of decreased bone formation, combined with stable bone resorption, was previously called senile osteoporosis by Riggs et al. [49]. Unfortunately, we were not able to assess bone formation markers in our cohort, which might have revealed whether there was indeed decreased osteoblast activity, making the case of senile osteoporosis more likely.

2. Sexual Dimorphism

Sex differences in medical research have recently received globally increased attention. Bone is one of the best investigated tissues for sexual dimorphism, but potential sex-differences in calcium and phosphate homeostasis have

been less-well studied [50]. Since a disbalance in calcium and phosphate homeostasis is associated with decreased bone health, these subjects are most definitely worthy of extended research. In chapter 2 we focused on sexual dimorphism in calcium and phosphate homeostasis and found, in a population-based study of elderly subjects, women above 45 years of age to have higher total serum calcium, as well as higher phosphate levels compared to men of the same age. Adjusting for the sex hormones estradiol and testosterone revealed differential effects: testosterone diminished the effect size for sex differences in serum phosphate but had no clear influence on sex differences in serum calcium. Estradiol did not influence the sex difference in either serum phosphate or calcium. Furthermore, data from the MrOs study showed an association between both higher estradiol and testosterone and lower levels of serum phosphate in aging men. Both these studies show that an important role for estradiol and testosterone in the calcium and phosphate balance may be anticipated, but to what extent and whether these influences are different between men and women remains to be further elucidated [51].
As mentioned before, the maintenance of adequate serum calcium and phosphate levels may be important for other conditions as well, such as cardiovascular diseases. There are clear sex differences in these conditions, as exemplified by several studies. A prevalence study with 883982 participants who were followed for a decade showed a higher prevalence of hypertension, hyperlipidemia, diabetes mellitus, and coronary artery disease in men compared to women [2]. However, there is an increase in incidence of cardiovascular diseases in women after menopause, when the incidence in women approaches the one in men [52-55]. It is unclear how alterations in sex hormones after menopause impact the occurrence of cardiovascular diseases. We observed an association with menopause and the evolution of sex differences in calcium and phosphate levels. Therefore, factors that underlie sex differences in calcium and phosphate homeostasis may also be important indicators for cardiovascular outcomes. Furthermore, osteoporosis and cardiovascular diseases appear to be associated as mentioned before. One study reported that women with osteoporosis, defined as having a BMD T-score < -2.5, have a 3.9-fold increased risk for cardiovascular events compared to women having a BMD with a T-score between -2.5 and -1 [56]. In a study by Campos-Obando et al., which used data from the Rotterdam Study, an association between lower BMD and a higher coronary artery calcification (CAC) score was found in women but not in men [57]. However, no association between higher CAC score and increased fracture risk was found in the

aforementioned study. In a meta-analysis by Ye et al., an association was found between lower BMD and atherosclerotic vascular abnormalities such as carotid artery calcifications and coronary artery disease [58]. However, a part of the associations between coronary artery disease and lower BMD or fracture risk can probably be explained by age and comorbid conditions such as diabetes mellitus, estrogen deficiency, hyperhomocysteinemia, and by common lifestyle factors such as physical inactivity and smoking [38, 56].

The need for scrutiny regarding sex differences in health and disease is obvious. However, much research outside of the osteoporosis field is predominantly performed in male subjects, both in animal and in human studies, which may lead to missing sex-specific effects [59, 60]. In order to understand sex-specific influences on homeostasis it is essential to understand male and female physiology. Assessment of differences in physiology may aid in the understanding of the underlying mechanisms of sex differences in various diseases. Moreover, this could help with sex-specific strategies in prevention and treatment of diseases such as osteoporosis and cardiovascular diseases.

3. Future perspectives

The results found in this thesis have provided new insights into calcium and phosphate homeostasis as well as the insight that failure to maintain adequate calcium and phosphate homeostasis may have an impact on both bone quality and on general health. Our findings also raise questions regarding the way in which to elaborate on our observations. There are several ways of approaching this in future research. Firstly, since the maintenance of calcium and phosphate homeostasis is part of a complicated and integrated system, whereby the hormones vitamin D, PTH and fibroblast growth factor (FGF23) play key roles, it is of interest to study these hormones in relation to the sex differences that arise with aging.

The maintenance of adequate serum ionized calcium levels in the body is tightly regulated by the parathyroid glands. When lower ionized calcium levels are detected via the calcium sensing receptor (CaSR), the parathyroid glands increase PTH secretion [61]. Chronically increased PTH levels, as in secondary hyperparathyroidism, result in pleiotropic effects. One of these effects is an increase in bone turnover via the RANKL/OPG system causing release of calcium and phosphate from the bone and reduced bone quality [62-64]. One would expect that the lower ionized calcium levels that we found in lactose intolerant people in chapter 5 would lead to secondary hyperparathyroidism, and could therefore reduce bone mineral density. However, in our

study, lactose intolerant subjects showed no difference in bone mass compared to controls, allowing one to question whether these subjects do indeed have secondary hyperparathyroidism. An alternative hypothesis could be that lactose intolerant people have a different setpoint of their CaSR, thereby diminishing the effects of lower calcium levels on BMD. The latter hypothesis would fit with findings in patients with Autosomal Dominant Hypocalcaemia (ADH), a condition characterized by low calcium levels but without a clear bone phenotype [65]. To study whether lower ionized and serum calcium levels in lactose intolerant subjects cause secondary hyperparathyroidism we need to address PTH levels.

Besides PTH, the phosphaturic hormone FGF23 coupled with the transmembrane protein Klotho, both associated with longevity in animal models, also play a role in the maintenance of serum calcium and phosphate levels [66-68]. FGF23 promotes phosphaturia by downregulating the Na-phosphate co-transporters (NaPi2a and NaPi2c) in the kidney, both by inhibiting 1,25-dihydroxyvitamin D_3 synthesis indirectly via suppression of CYP27B and by inhibition of PTH release [69]. Klotho is a co-receptor for FGF23 and *Klotho* deficient mice exhibit the same premature aging phenotype as *Fgf23* deficient mice, with higher levels of serum calcium, phosphate, and 1,25-dihydroxyvitamin D_3 [67]. In humans, malfunction of Klotho has been described in a case report of a girl with ectopic calcifications and increased serum calcium, phosphate, and 1,25-dihydroxyvitamin D_3 levels [70]. Loss of function mutations in *FGF23* cause autosomal dominant hypophosphatemic rickets [71, 72]. Since women have increased serum calcium and serum phosphate after menopause, it will be important to study whether Klotho and FGF23 levels alter around menopause as well. Sex differences in calcium and phosphate homeostasis stress the need to assess whether these hormones show sex-dependent differences during aging.

Another way to study the association between sex hormones and calcium and phosphate homeostasis will be to evaluate whether testosterone and estradiol have a direct effect on serum calcium and phosphate levels, or whether alterations in serum calcium and phosphate levels are merely the result of the uncoupling of bone formation and bone resorption. It is therefore important to evaluate whether sex hormones influence calcium and phosphate homeostasis throughout different stages in life, as the dynamics in bone metabolism differ from stage to stage. As stated in chapter 3, there are several ways to address the influence of estradiol and testosterone on serum calcium and phosphate homeostasis and their regulating hormones, such as PTH and

vitamin D. Assessing whether there are alterations in calcium and phosphate homeostasis in peripubertal boys and girls will help to elucidate whether there are sex differences at younger age as well. Moreover, women of reproductive age might show variations in their serum calcium and phosphate levels related to the hormonal fluctuations during their menstrual cycle. Additionally, the investigation of serum calcium and phosphate levels in patients that receive gonadotropin-releasing hormone agonists (GnRH-agonists) continuously in order to suppress luteinizing hormone (LH) and follicle stimulating hormone (FSH) production could yield new insights into the influence of sex hormones on calcium and phosphate homeostasis. Most suitable are patients that used the GnRH-agonists for non-malignant causes such as endometriosis and pubertas praecox. GnRH-agonists initially cause upregulation of LH and FSH, but with continuous use both are suppressed [73]. In order to address the effect of sex hormones, serum calcium, phosphate and their regulating hormones could be measured before they start treatment with a GnRH-agonist, during the first phase after initiation and after the initial upregulation when LH and FSH are suppressed. Furthermore, GnRH-agonists were shown to be associated with decreased BMD, and observational studies have demonstrated associations with cardiovascular mortality, making users of GnRH-agonists an interesting group to study with respect to the results described in this thesis [73, 74].

As stated previously, increased serum calcium and phosphate levels have been associated with cardiovascular diseases and mortality [37]. A next step to address calcium and phosphate homeostasis and their impact on cardiovascular disease could be to evaluate whether single nucleotide polymorphisms (SNPs), which influence serum calcium, phosphate levels and BMD, are associated with the occurrence of cardiovascular diseases in a so-called mendelian randomization design. Recently, Larsson et al. studied whether SNPs related to serum calcium levels were associated with coronary artery calcification and myocardial infarction. They found an increased risk for coronary artery disease associated with SNPs that predisposed to higher serum calcium levels [75]. A previous study by Babinsky et al. in 2015 did not find an association of polymorphisms in *CaSR* with vascular calcifications in renal transplant recipients [76]. However, they did find renal transplant recipients heterozygous for a polymorphism in the promoter region (rs115759455) of this gene to have increased serum phosphate levels. The main limitation of this study in establishing an association is the low number of subjects included. Only 284 subjects were included, and the heterozygous

variant of rs1157559455 was present in only 23 subjects, not allowing for the study of sex differences. The studies presented in this thesis, as well as some other studies in the field, suggest that sex differences exist in serum calcium and phosphate homeostasis after menopause [77-84].

Large cohort studies on cardiovascular events in both men and women are necessary to address whether genetic variants that influence calcium and phosphate levels are associated with either cardiovascular events, mortality, or fracture risk, or a combination of the three.

As stated before, following stratification into high and low DPD levels within osteoporotic postmenopausal women, our study on osteoclastic bone resorption revealed higher osteoclastogenesis and more osteoclastic bone resorption *in vitro* in osteoporotic subjects with high DPD levels. This leads to the question whether there are different types of osteoporosis present among these elderly women. Two previous studies on different types of osteoporosis in the elderly defined primary (type 1 osteoporosis) and senile osteoporosis (type 2 osteoporosis) [49]. Primary or type 1 osteoporosis predominantly affects women during aging and is thought to be related to the loss of estrogens, causing an increased loss of mainly trabecular bone, and to a lesser extend loss of cortical bone, resulting in vertebral fractures and Colles fractures, which occur at the ultradistal forearm, a site rich in trabecular bone. Senile osteoporosis or type 2 osteoporosis is considered a proportionate loss of cortical and trabecular bone, leading to hip and vertebral fractures, and is associated with aging in both men and women. It occurs in persons over 70 years of age and one of the mechanisms of senile osteoporosis is thought to be due to impaired production of 1,25-dihydroxyvitamin D_3 and decreased osteoblast activity while normal osteoclast activity is maintained [49, 85]. Other processes that are involved in the development of osteoporosis and fragility fractures with aging are increased oxidative stress, cell senescence, inflammation, osteocyte apoptosis, DNA damage, formation of advanced glycation end products, and a decrease in autophagy, mitochondria biogenesis, vascularity, hydration of bone, and alterations in musculoskeletal progenitor cells [86]. Recently, Ucer et al. studied the molecular basis of the way in which sex steroid deficiency as opposed to aging affects the skeleton in mice, and whether aging and sex steroid deficiency are two distinct mechanisms [87]. Both aging and sex steroid deficiency lead to accumulation of reactive oxygen species (ROS). In order to study whether there was a different molecular basis between aging and sex steroid deficiency the aforementioned researchers used aged mice which were either ovariectomized or gonadecto-

mized and compared them with aged transgenic mice with a genetic construct that enhanced catalase activity in either the myeloid or the mesenchymal lineage, thereby lowering the levels of ROS. Additionally, some of the transgenic mice were ovariectomized or gonadectomized as well, in order to discriminate between the influence of sex steroid deficiency and aging. They found that lower ROS levels in gonadectomized transgenic mice in the myeloid lineage prevented the mice from cortical but not from cancellous bone loss caused by sex steroid deficiency [87].

To date, people with osteoporotic fractures are not classified as suffering from either type 1 or type 2 osteoporosis, and first-line treatment is aimed at reducing osteoclast activity with bisphosphonates orally, intravenous zoledronic acid in case of intolerance, or other antiresorptive drugs such as denosumab [10]. Currently, there is no direct assessment of effectiveness of antiresorptive therapy possible with bisphosphonates, since changes in BMD are very modest and do not accurately reflect changes in fracture risk [88, 89]. Bone resorption markers could be used as a tool to assess adherence to antiresorptive therapy but not in order to assess which therapy should be instituted [90]. Our study in chapter 7 showed a clear association between high DPD levels and osteoclast activity in *in vitro* assays, and a low osteoclast activity in osteoporotic subjects with low DPD levels. It could be that subjects with low osteoclastogenic potential and low DPD levels have a low bone turnover phenotype. To reduce fracture risk in this subgroup, anabolic therapy could potentially be more beneficial than antiresorptive therapy, which could be favorable in the osteoporotic subjects with high DPD levels and high osteoclast activity. Assessing whether osteoporotic subjects with low DPD and osteoclastogenic potential benefit in a similar manner from antiresorptive therapy compared to osteoporotic subjects with high DPD levels may lead to alternative therapeutic strategies for these different groups. Discriminating between the types of osteoporosis based on a solid assay which evaluates osteoclast function in addition to the assessment of markers of bone resorption and bone formation might be helpful for patients, allowing them to receive more targeted therapy or to switch sooner to a different form of therapy. Such studies could potentially lead to increased fracture reduction, more personalized treatment, and improved quality of life.

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Summary

Bone is a dynamic tissue with several cell types that each have their own function. The main component of bone is hydroxyapatite which is a molecular structure that contains calcium and phosphate ions. Apart from being incorporated in hydroxyapatite these two ions are important in several processes in the body. Key regulators in calcium and phosphate homeostasis are the hormones vitamin D, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). A condition affecting bone is osteoporosis, which mainly occurs with aging and predominantly in women. The general aim of this thesis was to study calcium and phosphate homeostasis in relation to aging and the age-related disorder osteoporosis. **Chapter 1** presents the introduction and aim of the thesis.

Sex differences in serum calcium and phosphate have been reported but that to date their nature and underlying regulatory mechanisms have remained unclear.

Chapter 2 presents the data of a prospective population-based cohort study in total 9253 subjects from three independent cohorts of the Rotterdam Study (RS) on sex differences in calcium and phosphate levels. Furthermore, alkaline phosphatase (ALP), 25-hydroxyvitamin D_3 ($25(OH)D_3$), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), estradiol and testosterone as potential covariates to elucidate underlying mechanisms of sex differences were studied in this cohort. Women, compared to men, had significantly higher total serum calcium and phosphate. The potential covariates ALP, $25(OH)D_3$ or $1,25(OH)_2D_3$ could not be accounted for this sex difference. Adjustments for serum testosterone but not for estradiol diminished sex differences in serum phosphate levels. Pre-menopausal women had higher serum phosphate but not calcium compared to men but their serum phosphate levels were lower compared to postmenopausal women. In the total group, both serum calcium and phosphate levels decreased with age with a significant interaction for sex differences for serum calcium but not phosphate.

Chapter 3 shows the results of a study that examined whether a relation between sex differences in calcium and phosphate had a relation with age. In a total of 15211 samples from subjects derived from a clinical chemistry database from the Erasmus MC from three separate years, there was a significant sex*age interaction for serum calcium and phosphate levels and the calcium*phosphate product. Serum calcium, phosphate and the calcium*phosphate product were higher in women above 45 years of age compared to men of similar age. In two younger age groups no sex-differences

were found. In men, serum calcium and phosphate levels were highest in the youngest age group compared to the age groups of 18-44 and ≥45 years. In women, serum calcium levels were also significantly higher in the age group 1-17 and the age
group ≥45 years compared to the 18-44 years age group, while no significant difference was found between the highest and the youngest age group. In women, serum phosphate was different between the three different age groups with highest level in the group 1-17 years and lowest in the group 18-44 years.

Extreme phosphate levels have been associated with mineralization defects and increased fracture risk. Whether phosphate levels within the normal range are related to bone health in the general population is not well understood. In **Chapter 4** the results are described of an association study of phosphate with bone mineral density (BMD) and fracture risk in 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. This study showed that serum phosphate levels were 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]). Phosphate 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]). Phosphate was inversely related to lumbar spine BMD (LS-BMD) in men (ß: -0.06 [-0.11 to -0.02]) and not to femoral neck BMD (FN-BMD) in either sex. Adjustments were made for age, body mass index, smoking, serum levels of calcium, potassium, $25(OH)D_3$, estimated glomerular filtration rate (eGFR), FN-BMD, prevalent diabetes, and cardiovascular disease. Additional adjustments were made for phosphate intake, PTH, and FGF 23 levels in MrOS.

Chapter 5 presents the results of an association study between genetically defined lactose intolerance and body height, bone parameters and calcium homeostasis. 6367 individuals from the Rotterdam Study and 844 individuals from the Longitudinal Aging Study Amsterdam (LASA) were genotyped for genetically defined lactose intolerance, the C-variant of the T-13910C polymorphism upstream the lactase phlorizin hydrolase (LPH) gene. Furthermore, subjects were genotyped for vitamin D receptor (VDR) polymorphisms. Associations with body height ($p = 2.7x10^{-8}$) and vertebral area ($p = 0.048$) found in the Rotterdam study were explained by population stratification, as assessed by principal component analyses, and disappeared after additional adjustments. The C-allele of the T-13910C polymorphism causing lactose

intolerance was shown to be associated with lower dietary calcium intake ($p= 9.2x10^{-7}$) and serum calcium levels ($p = 0.02$) but not with BMD or fractures. For none of the parameters studied was interaction between the T-13910C polymorphism and VDR block 5 haplotype 1 observed. This study demonstrated the impact of population stratification and urges researchers to carefully take this into account in genetic associations, in particular, in dietary intake-related phenotypes, of which LPH and lactose intolerance are a strong example.

Peripheral blood mononuclear cells (PBMCs) are cells that are circulating in the blood with osteoclastogenic potential. Growth factors can stimulate these cells to differentiate into osteoclasts. Furthermore, these cells have 1α-hydroxylase activity and are therefore capable of converting $25(OH)D_3$ into $1,25(OH)_2D_3$. The ability of PBMCs to convert $25(OH)D_3$ into $1,25(OH)_2D_3$ is used in **Chapter 6** where a case of a young girl with pseudovitamin D deficiency is described with nearly absent 1α-hydroxylase activity in PBMCs compared to those of healthy blood donors. With DNA sequencing a new compound heterozygous mutation was found in the *CYP27B*1 gene; a (c413G>T) mutation in exon 3 (R138L) and a (c1232G>A) mutation in exon 8 (C411Y). *In silico* analyses confirmed that mutations at these positions are probably damaging for the protein since the amino acids are situated in a highly conserved region. Her healthy parents each of whom carried one of the mutations also had compromised conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ in PBMCs, being only marginally higher than the patient.

In **Chapter 7** the results are presented of a study on the osteoclastogenic potential of PBMCs derived from the blood of postmenopausal women with longstanding osteoporosis compared with the osteoclastogenic potential of PBMCs derived from the blood of healthy age-matched controls. This study showed no difference in PBMC osteoclastogenic capacity and activity between women with and without osteoporosis and at least one previous fracture, who were on average 29.5 years after menopause, suggesting that there is no difference in circulating osteoclast precursors. Stratification into high and low deoxypyridinoline (DPD) levels, a biomarker of bone resorption, showed higher osteoclastogenesis and more osteoclastic bone resorption in the high DPD group compared to the low DPD levels within the group of osteoporotic subjects.

Furthermore, this thesis focusses on TRPV5, which is a calcium channel and present in several tissues that play a role in calcium homeostasis like the osteoclast one of the cells types in the bone. In **Chapter 8** it is shown that a

life-long challenge of calcium homeostasis as present in Trpv5$^{-/-}$ mice caused accelerated bone aging and a low cortical and trabecular bone mass phenotype. Previous studies have shown that
Trpv5$^{-/-}$ mice display vitamin D resistance with aging at the level of the kidneys and intestines with hypercalciuria. Aging significantly increased serum $1,25(OH)_2D_3$ and PTH levels in both genotypes but they were more elevated in Trpv5$^{-/-}$ mice, whereas serum calcium was not affected by age or genotype. The phenotype of the Trpv5$^{-/-}$ mice suggests that maintenance of adequate serum calcium levels in patients with disturbances in calcium homeostasis should be a priority in order to prevent bone loss at older age. The Trpv5-deficient mouse appears to be a suitable model for lifelong challenge of calcium homeostasis and its consequences for bone metabolism.
In **chapter 9** this thesis is concluded by focusing on the integration of three main topics that have been discussed throughout the chapters; aging and its relationship with calcium and phosphate homeostasis, biomarkers and aging, and sexual dimorphism. Furthermore, suggestions for future research are given.

Samenvatting

Bot is een dynamisch weefsel dat bestaat uit verschillende celtypen die elk hun eigen functie hebben. Hydroxyapatiet is het hoofdbestanddeel van bot, calcium en fosfaat ionen zijn de belangrijkste bestanddelen van deze moleculaire structuur. Deze twee ionen zijn belangrijk voor verscheidene processen in het lichaam. Vitamine D, parathyroid hormoon (PTH) en fibroblast groei factor 23 (FGF23) zijn de belangrijkste hormonen die betrokken zijn bij het regelen van de calcium en de fosfaat homeostase. Osteoporose is een aandoen van het bot die voornamelijk bij het ouder worden voorkomt en vaker bij vrouwen dan bij mannen. Het doel van dit proefschrift was om de calcium en fosfaat homeostase te bestuderen in relatie tot veroudering en in relatie tot osteoporose. **Hoofdstuk 1** geeft een algemene inleiding en het doel van dit proefschrift.

Genderverschillen in serum calcium en fosfaat zijn reeds beschreven, desondanks is de reden van deze genderverschillen en de mechanismen die hieraan ten grondslag liggen nog onbekend. **Hoofdstuk 2** beschrijft de uitkomsten van een prospectieve populatie studie met 9253 deelnemers uit drie onafhankelijk cohorten van de Rotterdam studie (RS) naar genderverschillen in calcium en fosfaat spiegels. Om onderliggende mechanismen die aan de genderverschillen ten grondslag kunnen liggen te analyseren, werd de invloed van alkalisch fosfatase (ALP), 25-hydroxyvitamine D_3 ($25(OH)D_3$), 1,25-dihydroxyvitamine D_3 ($1,25(OH)_2D_3$), oestradiol en testosteron, zogenaamde co-variabelen, geanalyseerd. Vrouwen hadden vergleken met mannen hogere calcium en fosfaat spiegels. De potentiele co-variabelen ALP, $25(OH)D_3$ of $1,25(OH)_2D_3$ speelden geen rol bij deze genderverschillen. Adjusteren voor serum testosteron maar niet voor oestradiol verminderde de genderverschillen in serum fosfaat spiegels. Vrouwen die voor hun menopauze zaten hadden hogere serum fosfaat spiegels vergeleken met mannen, maar hun serum fosfaat spiegels waren lager dan die van vrouwen na de menopauzes. In de totale groep gingen zowel de serum calcium als de serum fosfaat spiegels met de leeftijd omlaag, met daarbij een significante interactie voor genderverschillen aangaande serum calcium spiegels maar niet voor fosfaat.

In **Hoofdstuk 3** worden resultaten beschreven van een studie naar leeftijd gerelateerde genderverschillen in calcium en fosfaat spiegels. In totaal werden 15211 personen geselecteerd uit een data base van het klinisch chemisch laboratorium van het Erasmus MC uit 3 verschillende jaren. In deze populatie werd een significante gender*leeftijd interactie gevonden voor serum spiegels van calcium en fosfaat en het calcium*fosfaat product. Serum spie-

gels van calcium en fosfaat en het calcium*fosfaat product waren hoger in vrouwen ouder dan 45 jaar in vergelijking tot mannen ouder dan 45 jaar. Er waren geen genderverschillen in de twee jongere leeftijdsgroepen die geanalyseerd werden. In mannen waren de serum spiegels voor calcium en fosfaat het hoogst in de jongste leeftijdsgroep in vergelijking tot de leeftijdsgroep 18-44 jaar en de leeftijdsgroep ouder dan 45 jaar. In vrouwen waren serum calcium spiegels ook significant hoger in de leeftijdsgroep 1-17 jaar en in de leeftijdsgroep ouder dan 45 jaar in vergelijking met de leeftijdsgroep vrouwen van 18-44 jaar, terwijl er geen significant verschil in serum calcium spiegels tussen de oudste en de jongste groep werd gevonden. Serum fosfaat spiegels in vrouwen waren verschillend tussen de drie leeftijdsgroepen met de hoogste waarde in de leeftijdsgroep 1-17 jaar en de laagste waarde in de leeftijdsgroep 18-44 jaar.

Extreme fosfaat spiegels zijn geassocieerd met mineralisatie defecten in bot en verhoogd fractuurrisico. Of fosfaat spiegels binnen de normaalwaarden gerelateerd zijn aan de gezondheid van bot in de algemene bevolking is niet bekend. In **Hoofdstuk 4** werd de associatie tussen fosfaat met bot mineraal dichtheid en fractuurrisico onderzocht in twee populatie gebaseerde cohorten: de Nederlandse Rotterdam Studie (RS-I, RS-II, RS-III; n=6791) en de Osteoporotische fractuur in mannen studie uit de Verenigde Staten (MrOS; n=5425). Deze studie laat zien dat serum fosfaat levels positief geassocieerd zijn met fractuurrisico in mannen en vrouwen van de RS-studie, deze bevindingen werden gerepliceerd in MrOS (gepoolde HR alle cohorten [95% CI]: 1.47 [1.31-1.65]). Fosfaat was geassocieerd met fractuurrisico in mensen met chronische nierziekte (CKD): (alle cohorten (1.44 [1.26-1.63]) en in mannen met CKD (1.93 [1.42-2.62]). Fosfaat was omgekeerd gerelateerd met lumbale wervelkolom bot minerale dichtheid (LS-BMD) in mannen (ß: -0.06 [-0.11 tot -0.02]) maar niet met bot minerale dichtheid van de femur nek (FN-BMD) in beide genders. Er werd gecorrigeerd voor leeftijd, body mass index (BMI), roken, serum calcium spiegels, kalium, 25(OH)D_3, geschatte glomerulair filtratie snelheid (eGFR), FN-BMD, prevalente diabetes mellitus en hart- en vaatziekten. Tevens werd gecorrigeerd voor fosfaat inname, PTH en FGF23 spiegels in MrOS.

Hoofdstuk 5 beschrijft de resultaten van een associatie studie tussen genetische gedefinieerde lactose-intolerantie en lichaamslengte, bot parameters en calcium homeostase. Genotypering voor genetische gedefinieerde lactose-intolerantie, de C-variant van het T-13910C polymorfisme gelegen voor het lactase phlorizine hydrolase (LPH) gen werd gedaan in 6367 deelnemers van

de Rotterdam Studie en 844 deelnemers van de Longitudinal Aging Study Amsterdam (LASA). Tevens vond genotypering voor vitamine D receptor (VDR) polymorfisme plaats. Een hoofdcomponent analyse liet zien dat de associaties tussen genetische gedefinieerde lactose-intolerantie en lichaamslengte ($p=2.7x10^{-8}$) en de associatie met wervelhoogte oppervlakte ($p=0.048$) in de Rotterdam Studie volledig konden worden toegeschreven aan populatiestratificatie. Er werd gevonden dat het C-allel van het T-13910C polymorfisme, wat lactose-intolerantie veroorzaakt, geassocieerd was met een lagere calcium inname via voeding ($p=9.2x10^{-7}$) en lagere geïoniseerde serum calcium spiegels (=0.02), maar niet met BMD of fracturen. Voor geen van de parameters die in deze studie geanalyseerd werden, werd er een interactie tussen het T-13919C polymorfisme en VDR blok 5 haplotype 1 gevonden. Deze studie laat duidelijk de invloed van populatie stratificatie zien en spoort wetenschappers aan om rekening te houden met populatie stratificatie, met name in voedingsgerelateerde fenotypes waar LPH en lactose-intolerantie voorbeelden van zijn.

Perifere bloed mononucleaire cellen, zijn cellen die in het circulerende bloed aanwezig zijn. Deze cellen kunnen door middel van groefactoren gestimuleerd worden om te differentiëren naar osteoclasten. Daarnaast bezitten deze cellen 1α-hydroxylase activiteit waardoor ze $25(OH)D_3$ kunnen omzetten in $1,25(OH)_2D_3$. In **Hoofdstuk 6** wordt doormiddel van deze techniek aangetoond dat de haar perifere bloed mononucleaire cellen van een jong meisje met pseudovitamine D deficiëntie een fors verlaagde capaciteit hebben om 25(OH)D3 om te zetten in $1,25(OH)_2D_3$ in vergelijking met gezonde bloeddonoren. DNA-analyses laten zien het bij dit meisje gaat om een nieuw samengestelde heterozygote mutatie; (c413G>T) in exon 3 (R138L) en een (c1232G>A) mutatie in exon 8 (C411Y). Software analyses bevestigden dat mutaties op deze posities in het DNA waarschijnlijk tot beschadigingen van het eiwit kunnen leiden aangezien de aminozuren zich in sterk geconserveerde gebieden van het DNA bevinden. Haar gezonde ouders, beide drager van een van de mutaties, hadden ook een verminderde omzetting van $25(OH)D_3$ naar $1,25(OH)_2D_3$ in perifere bloed mononucleaire cellen, deze was slechts marginaal hoger dan die van patiënte.

In **hoofdstuk 7** werd het potentieel van de perifere bloed mononucleaire cellen om zich tot osteoclast te ontwikkelen onderzocht bij vrouwen na de menopauze met osteoporose en vergeleken met die van gezonde leeftijd gepaarde vrouwen. De vrouwen waren gemiddeld 29,5 jaar na de menopauze. In deze studie werden geen verschillen gevonden in het osteoclast potentieel

van perifere bloed mononucleaire cellen tussen vrouwen met osteoporose en minimaal een fractuur, dit suggereert dat er geen verschil is in circulerende osteoclast voorloper cellen. Stratificatie van de vrouwen met osteoporose in hoge en lage deoxypyridinoline (DPD) spiegels, een biomarker van bot resorptie, liet een hogere osteoclast vorming en een toegenomen bot resorptie van de perifere bloed mononucleaire cellen *in vitro* zien in de vrouwen met de hoge DPD spiegels.

Dit proefschrift focust daarnaast ook nog op TRPV5, een calcium kanaal wat in verscheidene weefsels en celtypen waaronder osteoclasten, een van de celtypes in het bot, een rol speelt in de calcium homeostase. In **Hoofdstuk 8** wordt beschreven dat muizen die geen functioneel TRPV5 kanaal hebben, zogenaamde *Trpv5*$^{-/-}$ muizen, waarbij de calcium homeostase levenslang onder druk staat, een fenotype ontwikkelen van versnelde bot veroudering met daarbij een lagere corticale en trabeculaire bot massa. Voorgaande studies hebben laten zien dat *Trpv5*$^{-/-}$ muizen vitamine D resistent worden gedurende het leven op het niveau van de nieren en de darmen, resulterend in hypercalciurie. Veroudering verhoogde significant serum $1,25(OH)_2D_3$ en PTH spiegels zowel in de *Trpv5*$^{-/-}$ als de *Trpv5*$^{+/+}$ muizen, terwijl serum calcium spiegels niet beïnvloed werd door zowel de leeftijd als door het genotype van de muis. Het fenotype van de *Trpv5*$^{-/-}$ muis suggereert dat de handhaving van adequate serum calcium spiegels in patiënten met verstoringen in de calcium homeostase aandacht verdient om bot verlies op oudere leeftijd te voorkomen. De *Trpv5*-deficiente muis lijkt daarom een passend model te zijn voor onderzoek naar de consequenties van levenslange verstoringen in calcium homeostase en de consequenties hiervan op het bot metabolisme.

Hoofdstuk 9 is het laatste hoofdstuk van dit proefschrift, in dit hoofdstuk worden de drie belangrijkste onderwerpen die in de verschillende hoofdstukken aan bod komen; de relatie van calcium en fosfaat homeostase met veroudering, biomarkers en veroudering, en genderverschillen, geïntegreerd. Tevens worden er in dit hoofdstuk suggesties gedaan voor toekomstig onderzoek.

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Curriculum Vitae

Wera Nadia Hendrika Koek was born on October 15th, 1979 in Leeuwarden, the Netherlands. In 1998 she graduated from secondary school (VWO) at the Comenius College in Leeuwarden. From 1998 to 2005 she studied medicine at the Erasmus University Rotterdam. In her fourth year, she spent six months at ClÍnica Universidad de Navarra in Pamplona, Spain. There she worked as part of her fourth-year research project on establishing normal values for estimating body mass and fat mass with air-displacement plethysmography and bioelectrical impedance measurements. In 2005 she started working at the laboratory of the department of internal medicine at the Erasmus MC. She worked under supervision of Hans van Leeuwen for her PhD project until 2009. In 2009 she started with specialty training in Internal Medicine, first at the IJsselland Hospital in Cappelle aan den IJssel (supervised by dr. H.E van der Wiel) and from 2012 at the Erasmus MC (supervised by Prof. dr. J.L.C.M. van Saase and his successor dr. S.C.E. Klein Nagelvoort-Schuit). In 2013 she started a fellowship in vascular medicine (supervised by Prof. dr. E.J.G. Sijbrands) as part of her specialty training also at the Erasmus MC. In 2015 she became licensed as a specialist in Internal Medicine. From 2015 until 2017 she did a fellowship in Intensive Care medicine at the University Hospital of Maastricht (supervised by dr. D.C.J.J. Bergman). She is currently working as an Intensive Care Medicine specialist at the Medical Center Leeuwarden (MCL).

PhD Portfolio

Name:	Wera Nadia Hendrika Koek
Erasmus MC Department:	Internal Medicine
Research School:	Erasmus Postgraduate School Molecular Medicine (MolMed)
PhD Period:	August 2005-December 2008
Promotor:	Prof. dr. J.P.T.M. van Leeuwen and Prof. dr. M.C. Zillikens
Co-promotor:	Dr. ing. B.C.J. van der Eerden

PhD training

General academic skills

- Biomedical English writing 2007

In-depth courses

- ECTS Training Course: Genetic Aspects of Bone Disease 2006

(Inter)national conferences

- 15th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (Zeist, the Netherlands) 2005
- 33rd European Symposium on Calcified Tissues (Prague, Czech Republic) 2006
- 16th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (Zeist, the Netherlands) 2006
- 34th European Symposium on Calcified Tissues (Copenhagen, Denmark) 2007
- 17th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (Zeist, the Netherlands) 2007
- 35th European Symposium on Calcified Tissues (Barcelona, Spain) 2008
- 18th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (Zeist, the Netherlands)
- Molecular Medicine Day 2006, 2007
- Science Days Internal Medicine 2006, 2007, 2008

Presentations at (inter) national conferences

- "Evidence that a polymorphism in the lactase phlorizin hydrolase gene causes differences in height independent of current calcium intake" *European Symposium on Calcified Tissues 2005* -oral presentation

- "Gender differences in calcium homeostasis: a reflection of aging" *European Symposium on Calcified Tissues* 2006 -poster presentation:
- "Calcium: gender differences" *Annual Meeting of the Dutch Society for Calcium and Bone Metabolism 2007* - oral presentation:
- "Lifelong disturbance of calcium homeostasis in TRPV5 knock-out mice has profound effect on bone with ageing" *European Symposium on Calcified Tissues 2008* -poster presentation:

ErasmusMC Internal Medicine Seminars

- Endocrinology Lectures (monthly)
- Internal Medicine Departmental Seminar (weekly)
- Bone and Calcium Research Literature Discussion (monthly)
- Bone and Calcium Research Work Discussion (weekly)
- Bone and Calcium Research Lab Meeting (weekly)

Lecturing

Vaardigheids Onderwijs 2nd year medical students;

Hypothalamus-hypophysis-thyroid axis (2006, 2007)

Awards

2006 Young Investigator Award van de European Calcified Tissue Society