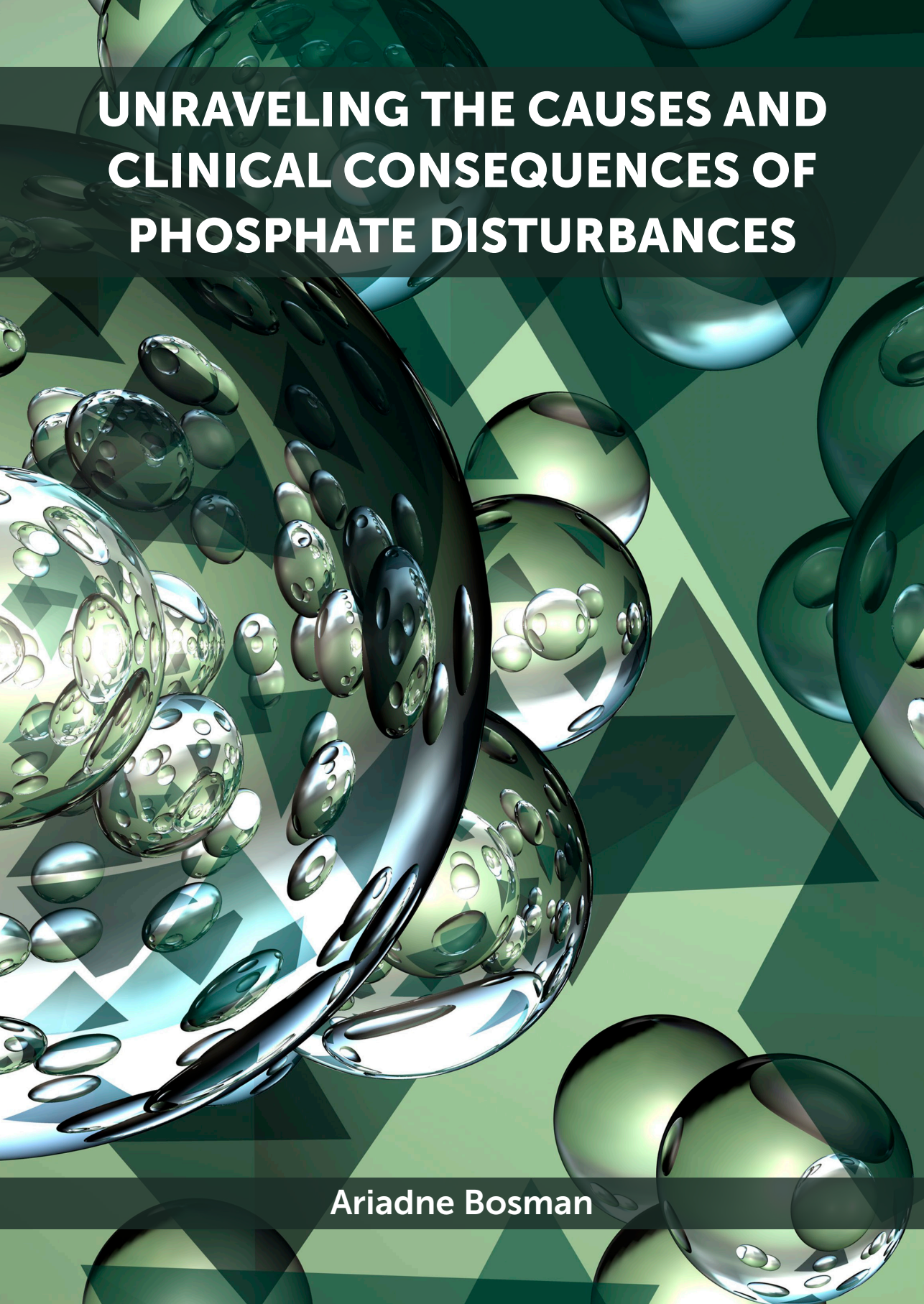
UNRAVELING THE CAUSES AND CLINICAL CONSEQUENCES OF PHOSPHATE DISTURBANCES



Ariadne Bosman

UNRAVELING THE CAUSES AND CLINICAL CONSEQUENCES OF PHOSPHATE DISTURBANCES

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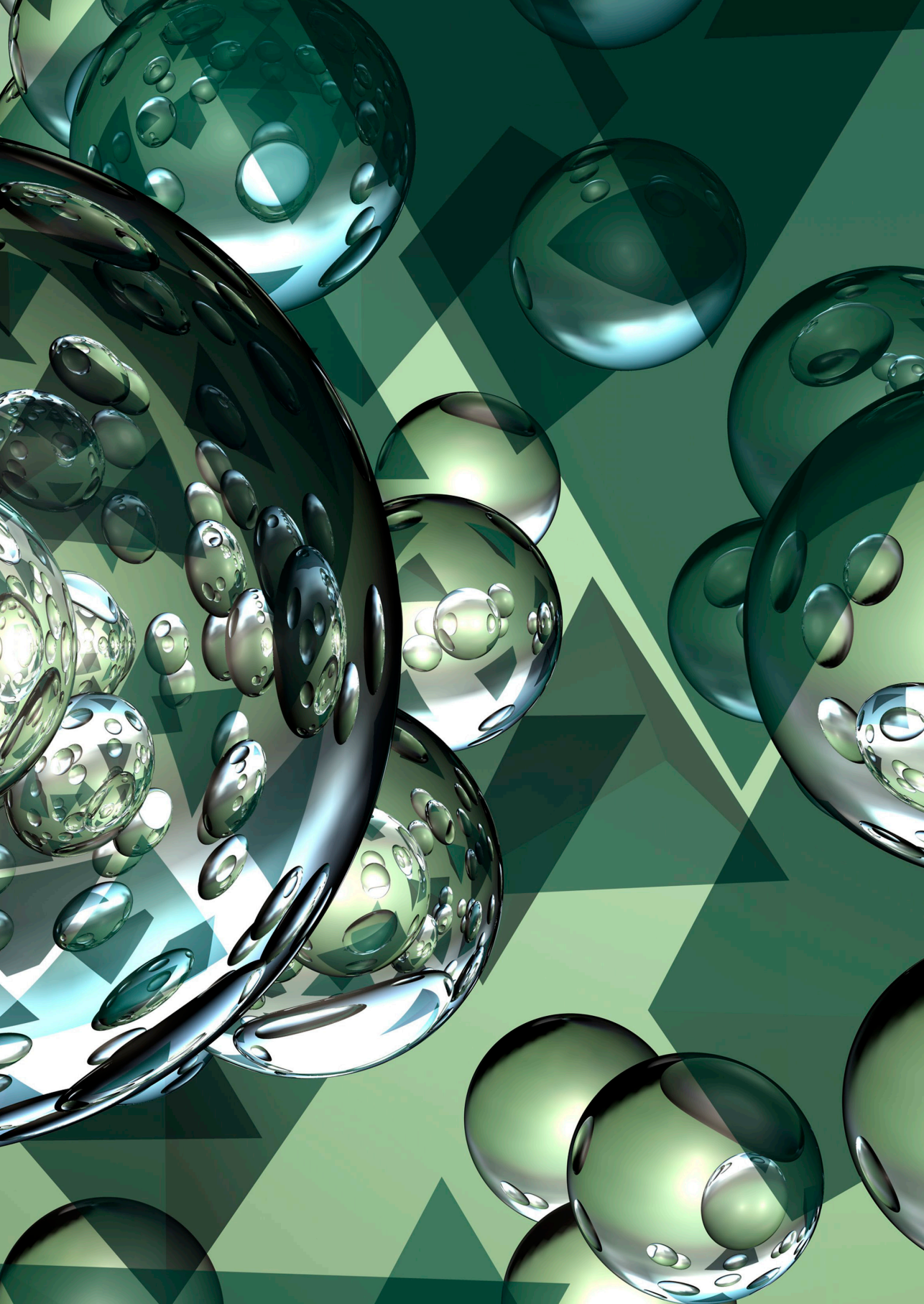

General introduction

1

PHOSPHATE IN NATURE

Phosphorus, with atomic number 15, is found in group 15 of the periodic table. It is highly reactive and therefore never found as a free element but it is present in all living organisms and essential for survival. Phosphorus is present in several forms: as inorganic phosphate, organic phosphate or as dimers (e.g., pyrophosphate) or polymers[2]. The difference between organic and inorganic phosphates is that organic phosphates contain carbon. Organic phosphates are esters of phosphoric acid bound to organic molecules. They play a key role in many cellular functions, e.g., as adenosine triphosphate (ATP) in energy metabolism, and as part of the structural framework of nucleic acids, including DNA and RNA[3]. Inorganic phosphates are salts of phosphoric acid and can be found in combination with sodium, potassium or calcium[2]. Together with calcium it forms hydroxyapatite, which is an essential crystal for the mineralization of bone[2]. Inorganic phosphate can be measured in biological fluids based on its reaction with a substance called ammonium molybdate. Together it forms a phosphomolybdate complex whose light absorbance at 340 nm is directly proportional to the amount of inorganic phosphate in a sample[4]. In this thesis, measurement of serum phosphate refers to the measurement of inorganic phosphate in serum.

PHOSPHATE IN HUMANS

Phosphorus is the second most abundant mineral in the human body[5]. In 1976, Marshall *et al*. studied the phosphorus composition in human plasma and found that 72% of the phosphorus content was comprised of organic phosphate while 28% was inorganic phosphate[4]. Most of the inorganic phosphate in humans, 85%, is stored as hydroxyapatite in the extracellular matrix of bone and teeth. Approximately 14% of the total inorganic phosphate load can be found within cells, where the concentration of free inorganic phosphate is equal to the extracellular inorganic phosphate concentration[2,4]. The remaining 1% is present in the extracellular fluids where it contributes to maintenance of the total body pH. In general, disturbances in intracellular inorganic phosphate concentrations influence cell metabolism and muscle function, whereas disturbances in extracellular inorganic phosphate concentrations may lead to the development of skeletal disorders and cardiovascular disease[2]. Phosphate related disorders will be discussed in more detail later in this chapter.

Our diet is the primary source of phosphate. The exact mechanisms of intestinal phosphate absorption are not completely understood, but it is known that sodium-dependent phosphate cotransporters type IIb (NPT2B, encoded by SLC34A2) and inorganic phosphate transporters type III (PIT, encoded by the SLC20 family) are

present in intestinal tissues[6,7]. Phosphate is excreted by the kidney but most is then reabsorbed from the urine in the proximal tubule by the sodium-dependent phosphate cotransporters type NPT2A and NPT2C [4].

PHOSPHATE HOMEOSTASIS

In general, a serum phosphate concentration in adults is considered normal when it lies between 0.80 mmol/L and 1.45 mmol/L.[8,9]. A serum phosphate concentration below 0.80 mmol/L is named hypophosphatemia and a serum phosphate concentration higher than 1.45 mmol/L is named hyperphosphatemia. These values concern fasting serum phosphate concentrations. Dietary intake leads to a postprandial increase in serum phosphate concentrations[10,11]. Serum phosphate concentrations are higher in infants most likely due to an increased need for phosphate for mineralization of the growing skeleton[8]. Serum phosphate concentrations decrease during infancy and adolescence. There is increasing evidence that serum phosphate concentrations also change during adulthood, which appears to be sex-specific[12,13]. However, this sex-specific age-related change in serum phosphate has not been studied in the general population and the mechanisms behind these changes are currently unknown.

The serum phosphate concentration is maintained by three major hormonal regulators: 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$), also known as calcitriol, parathyroid hormone (PTH) and fibroblast growth factor (FGF)23 (**Figure 1**)[2].

$1,25(OH)_2D$ is synthesized from vitamin D3, also known as cholecalciferol. Cholecalciferol is formed in the skin during exposure to sunlight, and can also be obtained from the diet. In the liver, cholecalciferol is converted to 25-hydroxy-vitamine D (25(OH)D) under the influence of several cytochrome P450 mixed-function oxidases (CYPs), e.g., CYP2R1 and others[14,15]. 25(OH)D can be measured in serum and is considered to be one of the most reliable biomarkers of vitamin D status. In the kidney, 25(OH)D is metabolised to the active $1,25(OH)_2D$ by CYP27B1, which encodes the enzyme 1α-hydroxylase. $1,25(OH)_2D$ regulates its own homeostasis by downregulating 1α-hydroxylase and by stimulating CYP24, which initiates the degradation of $1,25(OH)_2D$. $1,25(OH)_2D$ binds to the vitamin D receptor (VDR) that is e.g., present in the intestine, resulting in calcium and phosphate absorption from the diet[2,14,15].

PTH binds to the parathyroid hormone 1 receptor (PPR1), which is present in many tissues including osteoblasts, osteocytes, chondrocytes and proximal tubular cells. In the kidney, PTH downregulates the sodium-phosphate transporters (NPT2A and NPT2C), resulting in a decrease in renal reabsorption of phosphate. On the other hand, it induces

bone resorption through osteoblast-mediated activation of osteoclasts, thereby releasing phosphate as well as calcium. Moreover, it stimulates 1α-hydroxylase, which results in increased synthesis of calcitriol. However, the net effect is that serum phosphate levels go down[2,6].

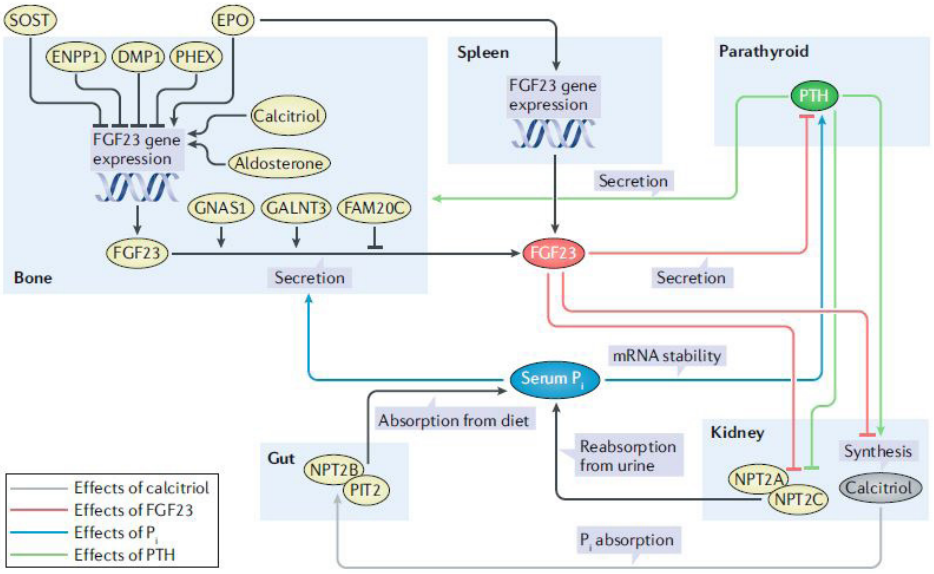

Figure 1. Phosphate homeostasis. Reprinted with permission from Nature Reviews Endocrinology[2]

In 2000, the ADHR consortium presented *FGF23* as the culprit gene in autosomal dominant hypophosphatemic rickets (ADHR)[16], although there had been a suspicion for the existence of a circulating phosphaturic factor for several years already[17,18]. The *FGF23* gene encodes a 251-amino acid long glycoprotein that requires post-translational O-glycosylation in order to be stabilized and phosphorylation to be cleaved[19,20]. FGF23 binds to the FGF receptor (FGFR) with the co-receptor α-klotho. In the kidney, FGF23 downregulates the abundance of NPT2A and NPT2C resulting in increased renal phosphate excretion, and it inhibits 1α-hydroxylase, thus leading to lower levels of $1,25(OH)_2D$ [6,20]. Knowledge of FGF23 regulation has increased substantially in recent years as shown in **Figure 2**. At the cellular level, dentin matrix protein 1 (DMP1) and phosphate regulating endopeptidase homolog X-linked (PHEX), suppress *FGF23* transcription[20]. N-acetylgalactosaminyltransferase 3 (GALNT3) is responsible for the O-glycosylation of FGF23, which prevents proteolytic processing, stabilizes the protein and allows the intact FGF23 to be secreted[6]. On the other hand, phosphorylation of FGF23 by the family with sequence similarity 20, member C (Fam20C), inhibits O-glycosylation by GALNT3 and

promotes FGF23 cleavage and inactivation by the subtilisin-like proprotein convertase furin[21]. FGF23 regulates serum phosphate concentrations, but phosphate in turn regulates FGF23 concentrations. Other factors that regulate FGF23 concentrations are circulating calcium, $1,25(OH)_2D$, PTH and α-Klotho , but also iron status, inflammation, erythropoietin, insulin and leptin[20,22-27]. Circulating FGF23 concentrations in humans can be measured by two different assays: the C-terminal FGF23 (cFGF23) assay and the intact FGF23 (iFGF23) assay. The cFGF23 assay detects both the full-length intact FGF23 and the C-terminal fragments that are released after cleavage. The iFGF23 assay detects only the intact FGF23[19].

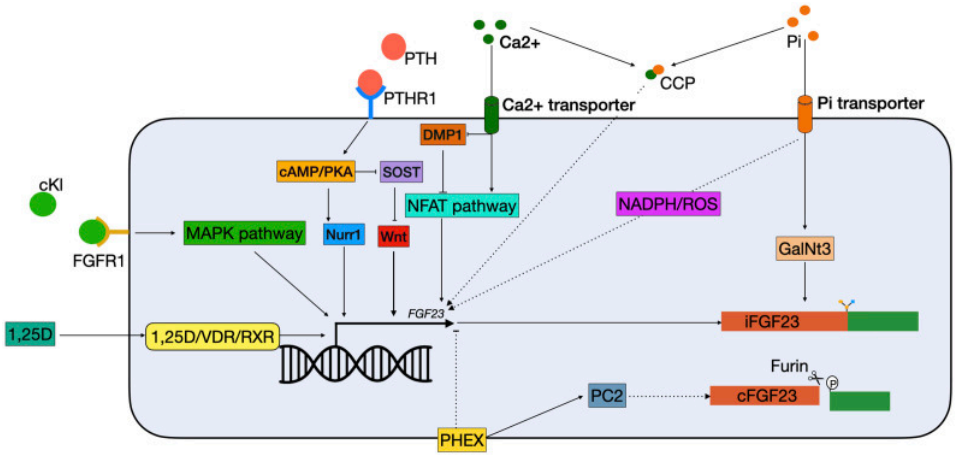

Figure 2. Overview of FGF23 regulation. Reprinted from Ratsma *et al*. Frontiers in Endocrinology [20]

$1,25(OH)_2D$, PTH and FGF23 are considered the major regulators of phosphate homeostasis but there are other factors affecting phosphate. Indirectly, serum phosphate concentrations can be affected by the aforementioned factors that regulate $1,25(OH)_2D$, PTH and FGF23. Other factors that regulate serum phosphate directly include estrogen, thyroid hormone and glucocorticoids. Estrogen and glucocorticoids decrease phosphate reabsorption in the kidney by decreasing NPT2A, whereas thyroid hormone increases NPT2A.[6] There is also evidence that potassium deficiency and metabolic acidosis increase urinary phosphate excretion[6]. Although knowledge is gradually increasing, phosphate homeostasis is not yet completely understood. Several studies have reported associations between serum phosphate and BMI but the pathophysiological mechanism underlying this relation is currently unknown[28,29]. Adiposity may influence phosphate homeostasis but serum phosphate or its regulators may also influence adiposity. Elucidation of this phenomenon is crucial for future studies on serum phosphate or BMI and their relation with morbidity and mortality.

HYPOPHOSPHATEMIA RELATED DISORDERS

Causes of hypophosphatemia can be divided into three groups, based on the underlying pathophysiological mechanism: hypophosphatemia due to a shift of phosphate from the extra- to the intracellular compartment, due to impaired intestinal phosphate absorption or due to increased renal excretion of phosphate [8]. Diagnostic algorithms for the workup of hypophosphatemia have been developed (**Figure 3**). Hypophosphatemia due to an intracellular shift of phosphate is usually observed in acute clinical settings such as diabetic ketoacidosis, respiratory alkalosis or as part of the refeeding syndroom[30-32]. However, studies have shown that hypophosphatemia is observed in ~20-25% of all ICU patients and that this is associated with renal phosphate loss in the majority of cases[33,34]. Acute severe hypophosphatemia can lead to neurological symptoms such as seizures and altered mental status, but also to impaired cardiac and respiratory function[32,35,36]. Although vital knowledge is lacking, hypophosphatemia in acute clinical settings is beyond the scope of this thesis.

Phosphate is pivotal for bone mineralization. Consequently, chronic hypophosphatemia can lead to rickets with the development of bone deformities in children and osteomalacia in adults[37]. Osteomalacia is a bone disease that is characterized by inadequate or delayed mineralization of the osteoid matrix synthesized by osteoblasts, resulting in an accumulation of unmineralized bone[38]. Chronic hypophosphatemia can be caused by (mono)genetic disorders, but it can also develop during the course of life e.g., due to the formation of an FGF23-producing tumor or due to medication use. Hypophosphatemia due to impaired intestinal absorption can also be caused by (mono)genetic disorders or it can be acquired.

Vitamin D-dependent rickets type IA (VDDR-1A) is caused by an inactivating mutation in the *CYP27B1* gene (encoding the aforementioned 1α-hydroxylase), resulting in low levels of $1,25(OH)_2D$, hypocalcemia, increased PTH and hypophosphatemia with normal levels of 25(OH)D [39]. Hereditary vitamin D-resistant rickets (HDVRR), caused by mutations in the vitamin D receptor gene leads to end-organ resistance to $1,25(OH)_2D$. This results in hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and elevated $1,25(OH)_2D$ concentrations[40,41]. Acquired causes of hypophosphatemia due to impaired intestinal absorption include impaired dietary intake, use of phosphate binders and malabsorption[42,43].

Hypophosphatemia resulting from increased renal excretion can be further divided in FGF23-mediated and non-FGF23-mediated hypophosphatemia. The most common monogenetic form of FGF23-mediated hypophosphatemia is X-linked hypophosphatemia (XLH), caused by inactivating mutations in the PHEX gene leading to increased circulating FGF23 concentrations and hypophosphatemia[44,45]. XLH is characterized by rickets in children,

which includes longitudinal growth delay and development of bone deformities, and osteomalacia in adults. In addition, patients often develop dental problems, fractures, early-onset osteoarthritis, hearing loss and enthesopathy[46,47]. Conventional treatment consists of oral phosphate and active vitamin D supplementation. Recently, a new treatment for XLH has become available for both paediatric and adult patients: the anti-FGF23 antibody burosumab[48,49]. Burosumab shows greater clinical improvement in rickets severity, growth and biochemical abnormalities than conventional therapy[48]. Reports on the prevalence of XLH range between 1:20,000 and 1:60,000[50-52], but the prevalence and disease course of XLH and other causes of chronic hypophosphatemia in the Netherlands is currently unknown. For this reason, the observational registry for genetic hypophosphatemia and acquired renal phosphate wasting in The Netherlands (ORPHOS-NED) has been set up. The rationale and design of ORPHOS-NED will be discussed at the end of this chapter.

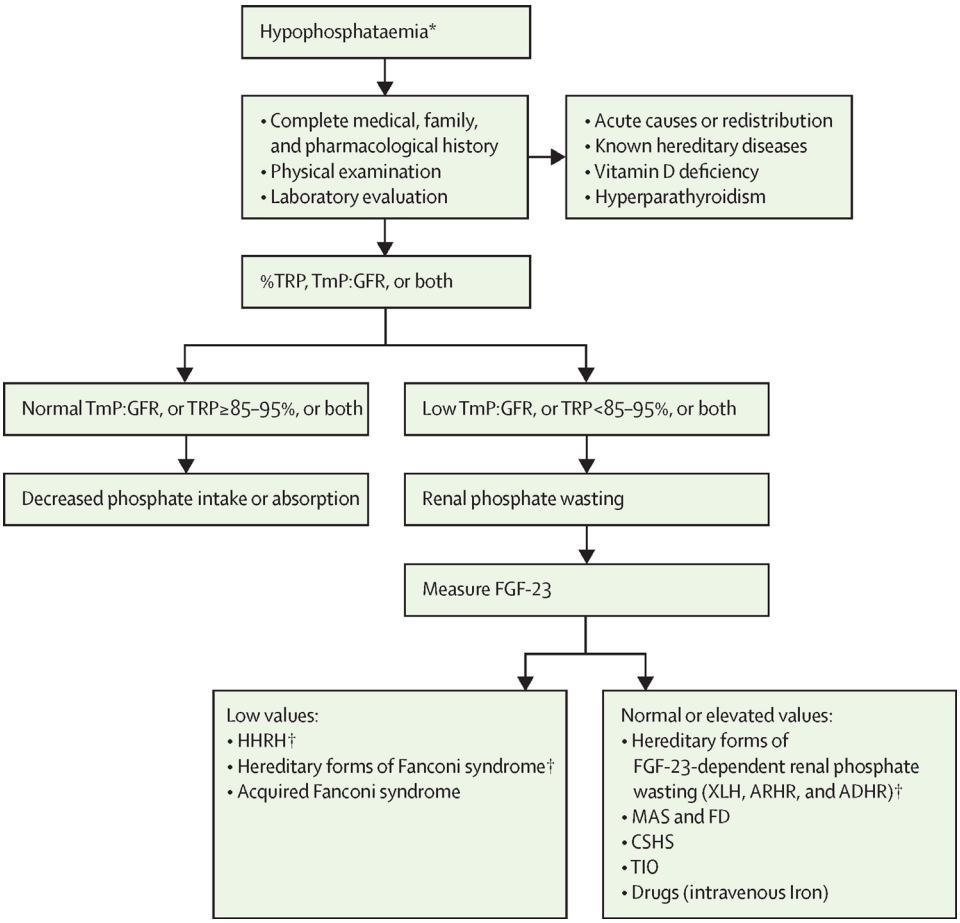

Figure 3. Diagnostic algorithm of hypophosphatemia. Reprinted from Florenzano et al. Lancet Diabetes Endocrinology[1]

Other monogenetic forms of FGF23-mediated hypophosphatemia are autosomal dominant hypophosphatemic rickets caused by a mutation in the FGF23 gene, and autosomal recessive hypophosphatemic rickets caused by mutations in Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) or DMP1[16,53,54]. Increased FGF23 production leading to hypophosphatemia can also occur in fibrous dysplasia lesions, and epidermal nevi leading to cutaneous skeletal hypophosphatemia syndrome[55,56].

A rare and largely underdiagnosed cause of acquired FGF23-mediated hypophosphatemia is tumor-induced osteomalacia (TIO). This paraneoplastic syndrome is caused by a FGF23-producing tumor that arises from mesenchymal tissue[57,58]. The tumor can develop anywhere in the body but it can be very small and therefore difficult to identify by imaging[59]. The disease can be suspected based on a combination of hypophosphatemia from renal phosphate wasting, reduced or inappropriately normal $1,25(OH)_2D$ concentrations and increased FGF23 concentrations[60,61]. Previous studies have investigated the clinical characteristics of TIO, but reviews also included cases of suspected TIO, when a tumor had not been identified or could not be resected, leaving open the possibility of alternative diagnoses[60,61].

Use of certain drugs have also been associated with FGF23-mediated hypophosphatemia, including ferric carboxymaltose infusions and corticosteroids. Ferric carboxymaltose infusions cause an increase in iFGF23 concentrations leading to hypophosphatemia[62,63]. Corticosteroids have been suggested to be among the most common drugs associated with hypophosphatemia in hospitalized patients[64]. Corticosteroids can cause hypophosphatemia by decreasing intestinal absorption and renal tubular reabsorption of phosphate[65], a process that may be mediated by FGF23[66]. Much less is known about the occurrence of hypophosphatemia in patients with Cushing's syndrome (CS), a disease characterized by hypercortisolism[67,68]. Several case reports have described hypophosphatemia in patients with CS, and a small cohort study in seven patients with CS reported an increase in serum phosphate after treatment for CS[69-71]. However, the prevalence of hypophosphatemia in CS is unknown and the pathophysiological mechanism is not very well understood.

An example of monogenetic non-FGF23-mediated hypophosphatemia is hereditary hypophosphatemic rickets with hypercalciuria (HHRH). HHRH is a disease caused by a mutation in *SLC34A3*, encoding NPT2C. HHRH is characterized by renal phosphate wasting, hypophosphatemia and rickets accompanied by elevated levels of $1,25(OH)_2D$ and increased urinary calcium excretion[72]. Another important cause of non-FGF23-mediated renal phosphate wasting is Fanconi syndrome. Fanconi syndrome is a generalized proximal tubulopathy of the kidney resulting in renal phosphate wasting and hypophosphatemia, but also in loss of e.g., glucose, bicarbonate and urate[73].

It can be caused by mutations in *SLC34A1* or *OCRL1* (Lowe syndrome)[74,75], but it can also develop later in life due to multiple myeloma or certain drug treatments, such as some antiretrovirals, chemotherapeutics and antiepileptic drugs[76,77]. Thiazide and loop diuretics have also been suggestively linked to hypophosphatemia. It was hypothesized that thiazide and loop diuretics affect serum phosphate concentrations by inhibition of carbonic anhydrase and induction of hypokalemia[78-81]. Studies on thiazide and loop diuretics have been conducted in specific patient groups including hospitalized patients and patients with congestive heart failure[64,82]. The effect of loop or thiazide diuretics use on serum phosphate levels in the general population and the prevalence of hypophosphatemia in loop or thiazide diuretics users is currently unknown.

HYPERPHOSPHATEMIA RELATED DISORDERS

While conditions of low phosphate are characterized by rickets or osteomalacia, conditions of high phosphate present with ectopic calcifications. Familial tumoral calcinosis is a monogenetic disease caused by a mutation in either *GALNT3*, *FGF23* or *KL*, encoding the co-receptor klotho[83]. The disease is characterized by hyperphosphatemia, an increased calcium x phosphate product and ectopic calcifications. These calcification can develop in the skin and subcutaneous tissue, but also in the eyelids and/or conjunctivae, and in small and large blood vessels.

The kidney is a major player in phosphate homeostasis. Most of the body's phosphate excess is excreted via the urine. In the setting of chronic kidney disease (CKD), with deteriorating glomerular filtration rate, the kidney's ability to excrete phosphate will also decrease[4,84]. Consequently, hyperphosphatemia is a common finding in patients with chronic kidney disease[84]. Several studies have shown that increasing serum phosphate concentrations are associated with vascular calcifications and increased mortality risk in patients with CKD[85,86]. There is increasing evidence that serum phosphate is also related to cardiovascular disease in the non-CKD population, but whether serum phosphate is causally related to coronary artery calcification in the general population is unknown[87-89].

MENDELIAN RANDOMIZATION

All genetic information is stored in our DNA (=deoxyribonucleic acid). DNA contains all the information that is vital for development, function and maintenance of an organism. It is composed of two strands that together form a double helix. The strands consist of sugar-phosphate molecules to which one of four nucleotides is bound: Adenine (A), Thymine (T), Cytosine (C) or Guanine (G). Each strand holds a sequence of these nucleotides[90],

which is for ~99.5% similar between any two humans but the variations in the sequence make that every human being is unique. The 1,000 Genomes Project developed a reference human genome and found that one individual's genome differs from this reference human genome at 4.1 million to 5.0 million sites[91]. The most common genetic variation in the human genome is the Single Nucleotide Polymorphism (SNP). A genome-wide association study (GWAS) is designed to detect associations between SNPs and a trait of interest[92]. The goal of a GWAS is to understand biological and pathophysiological mechanisms. To date, two GWASs on serum phosphate have been published[93,94]. The SNPs identified in a GWAS can be used in Mendelian randomization (MR) studies. MR has been applied in several studies in this thesis. MR is a method that utilizes natural genetic variation (SNPs) to mimic randomized controlled trials. Epidemiological studies are susceptible to confounding, reverse causation and various biases. MR is less prone to confounding and unaffected by reverse causation, because the genetic predisposition of an individual for a trait is already present at conception[95,96]. For this reason, MR can be used to test causality of phenotypic associations. MR relies on three assumptions: 1. the genetic instrument is associated with the exposure under study, 2. the genetic instrument is associated with the outcome only through the exposure, 3. the genetic instrument is not associated with potential confounders (**Figure 4**)[95].

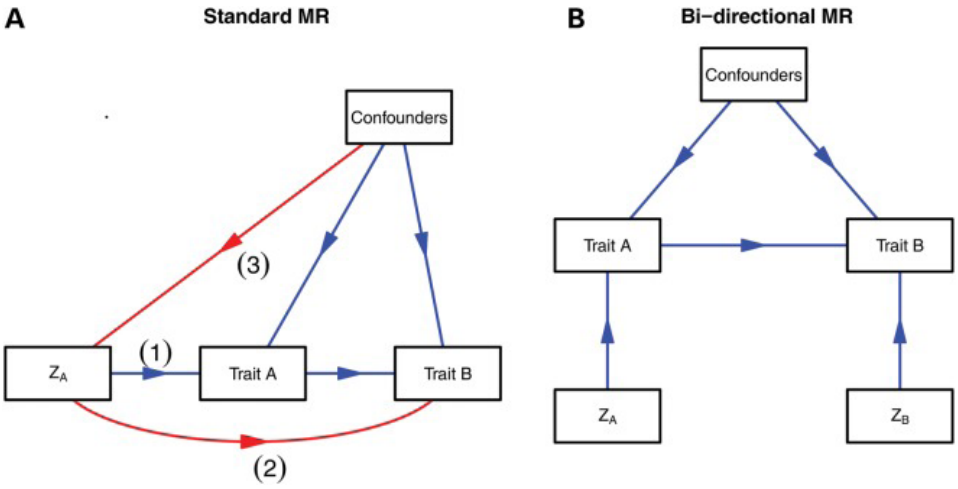

Figure 4. Schematic representation of MR. (A) Mendelian randomization can be used to test the hypothesis that trait A causes trait B, provided that conditions (1), (2) and (3) are met adequately. Reprinted from Davey Smith *et al*. Human Molecular Genetics [95].

STUDY POPULATIONS IN THIS THESIS

Three datasets have been used for analyses in this thesis: the Rotterdam Study (RS), the UK Biobank (UKBB) and the ORPHOS-NED study.

The Rotterdam Study is a population-based cohort study of men and women age 45 years or more living in the Ommoord district in Rotterdam, the Netherlands. The study commenced in 1990 with 7,983 participants in the first cohort, named RS-I. In 2000, 2005 and 2017 study cohorts RS-II, RS-III and RS-IV followed, resulting in ~18,000 participants. Participants have been interviewed and examined at baseline and during several follow-up visits every three to six years[97]. All participants provided written informed consent to participate in the study and to have their information shared by treating physicians. Non-fasting serum phosphate concentrations have been measured in the baseline visit of RS-I (RS-I-1) and fasting serum phosphate has been measured in the third visit of RS-I (RS-I-3) and in the baseline visits of RS-II and RS-III.

UK Biobank (UKBB) is a major biomedical database containing over half a million participants. These participants were between 40 and 69 years old when they were recruited between 2006 and 2010 in 22 assessment centers throughout the United Kingdom. Similar to RS, participants consented to collection and storage of genetic data and data on lifestyle and health[98]. Non-fasting serum phosphate concentrations have been measured at baseline.

The observational registry for genetic hypophosphatemia and acquired renal phosphate wasting in the Netherlands (ORPHOS-NED) is a nationwide observational cohort study in adult and paediatric patients with chronic hypophosphatemia. ORPHOS-NED was set up by Prof. M. C. Zillikens and Dr. B. C. J. van der Eerden from the Erasmus MC, in collaboration with (paediatric) endocrinologists and nephrologists from all over the Netherlands. The goal of this study is to evaluate demographic, biochemical, radiological and genetic characteristics, treatment and quality of life of patients with different forms of chronic hypophosphatemia by performing medical chart reviews and sending out quality of life questionnaires. The study was initiated in 2020 and currently includes ~150 patients from nine hospitals, including eight academic hospitals.

OUTLINE AND AIM OF THIS THESIS

The aim of this thesis is to examine causes and clinical consequences of phosphate disturbances. In *Part I*, the sex- and age-related differences in serum phosphate are investigated. In **Chapter 2**, age- and sex-related differences in serum phosphate in the

general population are investigated. **Chapter 3** explores the influence of vitamin D and sex hormones on the sex differences in serum calcium and phosphate. *Part II* focuses on associations between serum phosphate and BMI and between serum phosphate and diuretic use in the general population. **Chapter 4** describes the results of a Mendelian randomization study on the causality of associations between BMI and serum phosphate. In **Chapter 5**, associations between serum phosphate and use of loop and thiazide diuretics in the general population are investigated. In *Part III,* several causes of phosphate disturbances are examined. In **Chapter 6**, the results of a systematic clinical review on 895 published cases of tumor-induced osteomalacia are presented. Then, in **Chapter 7**, the prevalence of hypophosphatemia is investigated in patients with Cushing's disease. **Chapter 8** describes a patient with hypophosphatemia, elevated C-terminal FGF23, but normal intact FGF23, illustrating the limitation of C-terminal FGF23 assays to diagnose FGF23-related disturbances. *Part IV* focuses on consequences of phosphate disturbances, with a description of Dutch X-linked hypophosphatemia patients from the ORPHOS-NED study in **Chapter 9,** and the results of a Mendelian randomization study on the causality of association between serum phosphate and coronary artery calcification in **Chapter 10**. Finally, in **Chapter 11**, I discuss the results obtained by the studies in this thesis, focussing on the importance of creating awareness among health care professionals about the relevance of abnormal serum phosphate levels and of phosphate related disturbances.

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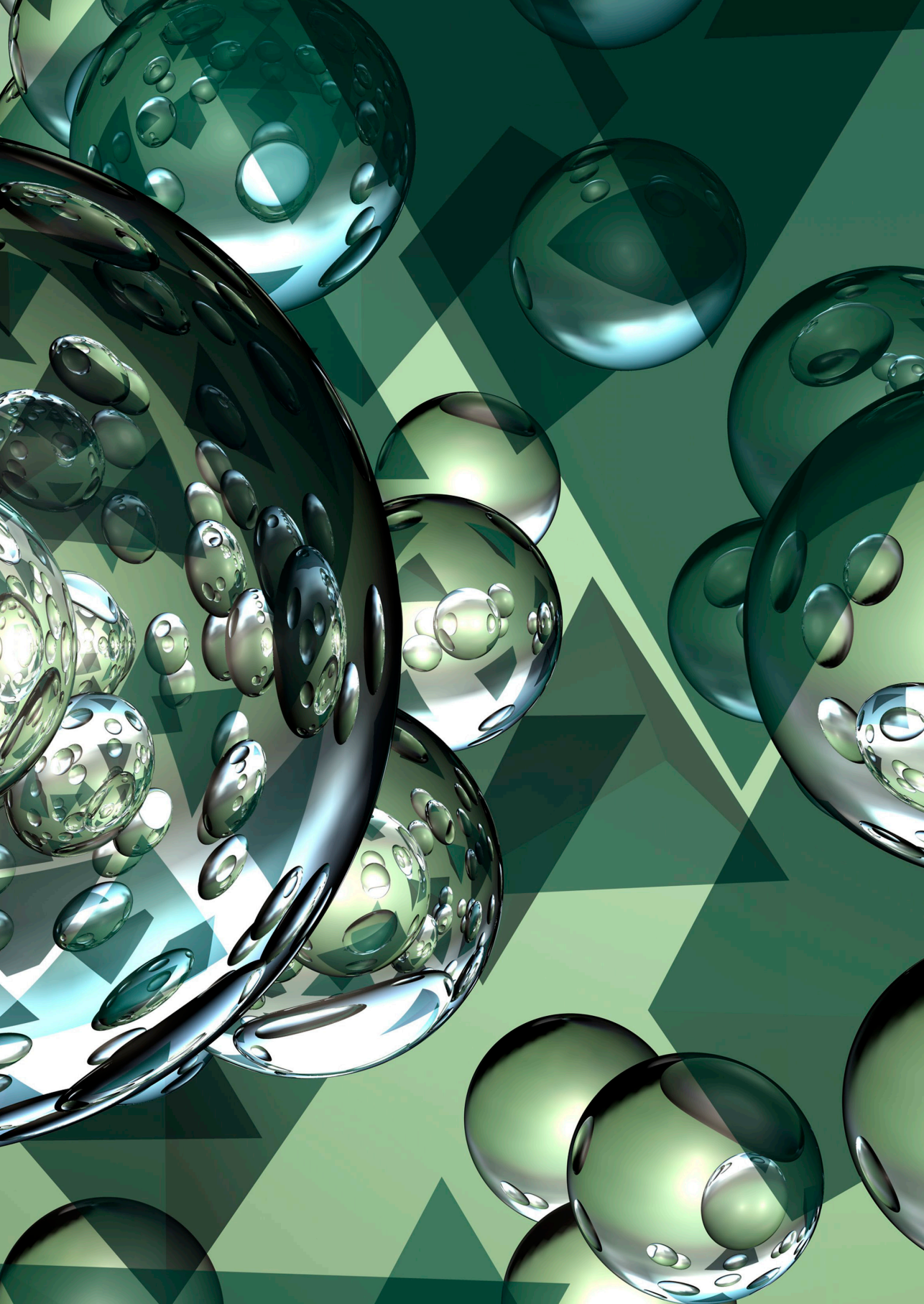
PART I

Sex- and age-related differences in serum phosphate in the general population



Serum phosphate in the general population: a need for sex-specific reference intervals

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Submitted

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ABSTRACT

Background

Phosphate is important for several metabolic functions and essential for bone mineralization. Sex-differences exist in the relation between serum phosphate and certain diseases. The reference interval for phosphate is age-adjusted in infants, but most institutions use the same intervals for adult men and women despite increasing evidence for age and sex-differences. We aimed to study these differences in two large population-based cohorts in order to evaluate whether current reference intervals are adequate.

Methods

8837 participants from three cohorts of the Rotterdam Study (RS) and 422,443 participants from UK Biobank (UKBB), aged 40 and older and without kidney impairment, were analyzed for sex-differences in serum phosphate using standard reference values (0.8-1.45 mmol or 2.5–4.5 mg/dL). Analyses were further stratified in women by menopausal status.

Results

Women had higher serum phosphate concentrations and a higher population range compared to men in all cohorts. Hypophosphatemia was more prevalent in men and hyperphosphatemia was more prevalent in women. Sex-differences were present in all age-categories. Perimenopausal women had higher serum phosphate concentrations than men of the same age, but lower than postmenopausal women of the same age.

Conclusions

This study in two population-based cohorts showed that women have higher serum phosphate concentrations than men and that women show a marked increase in serum phosphate during menopause. Moreover, the population range for serum phosphate was higher in women than in men. We propose to introduce sex-specific reference intervals for serum phosphate in adults older than 45 years.: 0.75–1.35 mmol/L in men and 0.85–1.45 mmol/L in women.

INTRODUCTION

Phosphate is important for several metabolic functions and together with calcium it forms hydroxyapatite, which is essential for bone mineralization[1]. Homeostasis is maintained primarily by parathyroid hormone (PTH), 1.25-dihydroxy-vitamin D ($1.25(OH)_2D$) and fibroblast growth factor (FGF)23[1,2]. In adults, hypophosphatemia is defined as a serum phosphate concentration below 0.8 mmol/L or 2.5 mg/dL (conversion factor 3.125)[3]. Hyperphosphatemia is generally defined as a serum phosphate concentrations above 1.45 mmol/L or 4.5 mg/dL[4]. The reference interval for serum phosphate is higher for infants and gradually decreases during infancy and adolescence[3].

Hypophosphatemia can lead to fatigue and muscle weakness and to impaired bone mineralization resulting in rickets in children and osteomalacia in adults[3]. The most common monogenetic cause of chronic hypophosphatemia is X-linked hypophosphatemia (XLH). XLH is caused by a mutation in the PHEX gene, resulting in increased FGF23 levels, which stimulates renal phosphate wasting. Acquired hypophosphatemic disorders include tumor-induced osteomalacia (TIO) or medication induced hypophosphatemia[5,6]. In contrast, hyperphosphatemia can lead to calcifications. Extreme extra-skeletal calcifications can be seen in hyperphosphatemic familial tumoral calcinosis[5]. Several studies have shown that serum phosphate is associated with an increased risk for cardiovascular disease and mortality in patients with CKD[7,8]. Moreover, phosphate has been associated with coronary artery calcification, microvascular dysfunction and increased risk of end stage renal disease and all-cause and cause-specific (CVD and COPD) mortality in the general population without CKD, even with serum phosphate in the currently accepted normal range[9-12]. Interestingly, many of these studies have found sex differences in the associations between phosphate and adverse outcomes, with mostly stronger associations in men[9,10].

The reference interval for phosphate is age-adjusted in infants, but much less is known about age-related changes in serum phosphate later in life. Cirillo et al. reported an age-related decline of serum phosphate levels, with a transient increase in women during the perimenopausal period, in a population study of 2107 men and 2560 women[13]. Koek et al. found similar results using a hospital information system: women above the age of 45 years had higher serum phosphate levels compared to men[14]. These findings, together with the reports of sex differences in the associations between phosphate and adverse outcomes, raise the question whether the current reference interval for serum phosphate is appropriate for both sexes at all ages. In this study, we analyzed age-related sex differences in serum phosphate concentrations and prevalence of hypophosphatemia and hyperphosphatemia in two large population based cohorts: the Rotterdam Study (RS) and in UK Biobank (UKBB).

METHODS

All analyses were performed in participants of RS and UKBB without CKD, defined as a eGFR >60 ml/min/1.73m^2, because phosphate excretion takes place primarily in the kidney and patients with CKD develop hyperphosphatemia. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine concentrations[15]. While literature often refers to 'serum' phosphate as a measure for the circulating phosphate concentration, many laboratories use lithium heparin plasma as a matrix. In light of this, the reference intervals for phosphate in serum are interchangeable with those in lithium heparin plasma (data not shown).

The Rotterdam Study

The Rotterdam Study (RS) is a prospective cohort study of men and women aged 40 years or more, conducted in the district of Ommoord, Rotterdam. Rationale and design have been described elsewhere[16]. RS is composed of four cohorts named RS-I, RS-II, RS-III and RS-IV, initiated in 1989, 2000, 2006 and 2016 respectively. Taken together, RS is composed of almost 18.000 participants. After recruitment, participants are assessed at baseline and during several follow-up visits. The current study includes participants from the second follow-up visit of RS-I (RS-I-3, from henceforth referred to as RS-I) and the baseline visits of RS-II and RS-III, with fasting serum phosphate and creatinine measurements. Serum phosphate concentrations were measured using a method that is based on the formation of ammonium phosphomolybdate, a compound that is directly proportional to the inorganic phosphate concentration. Information on menopausal status was collected during home interviews[16]. Female participants were asked about the timing of the last menstrual period. Women were considered postmenopausal when they had not had a menstrual period for 12 months.

UK biobank

UK biobank is a major biomedical database with over half a million participants aged between 40-69 years when recruited in 2006-2010 in 22 assessment centers throughout the UK. Participants consented to collection and storage of genetic, lifestyle and health information[17]. Serum phosphate and creatinine concentrations were measured regardless of fasting status at two instances: during the initial assessment visit between 2006 and 2010, at which participants were recruited and consent was given; and during the first repeat assessment visit between 2012 and 2013. For the current study, we analyzed data from the initial assessment visit. Serum phosphate concentrations were measured by phosphomolybdate complex analysis (Beckman Coulter AU5800, Beckman Coulter UK, Ltd). At the initial assessment, female participants were asked whether they had had their menopause. Women that answered yes to this question were considered postmenopausal.

Statistics

Continuous measures are reported as mean (standard deviation (SD)). Categorical measures are reported as percentages. RS cohorts were combined for the analyses of age categories and menopause. For the analyses with age categories, we stratified subjects in consecutive decades. Differences between men and women were tested using independent T-test for continuous measures and chi-square test for categorical measures. Differences in serum phosphate between the different age categories were tested using one-way ANOVA. Outliers of more than 6 standard deviations were excluded from analysis. The population range is defined as the range that includes the values of 95% of the population under study. Hypophosphatemia was defined as a serum phosphate level below 0.80 mmol/L (2.5 mg/dL)[3]. Hyperphosphatemia was defined as a serum phosphate level higher than 1.45 mmol/L (4.5mg/dL)[4]. All analyses were performed with IBM SPSS software, version 28.0.0.0 and R version 3.6.1 (Vienna, Austria).

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalogue number NL6645 / NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. UK Biobank has been approved by the North West Multi-centre Research Ethics Committee. UKBB data were accessed through application 48264.

RESULTS

Study population

A total of 3,214 participants from RS-I, 2,309 participants from RS-II and 3,314 participants from RS-III with an eGFR >60ml/min/1.73m^2 had information on age and serum phosphate and were included for analysis. Mean (SD) age was 71.5 (76.7) years in RS-I, 63.9 (7.2) years in RS-II and 56.7 (6.4) years in RS-III. Mean (SD) serum phosphate was 1.11 (0.16) mmol/l in RS-I (population range: 0.79–1.42 mmol/l), 1.08 (0.16) mmol/l in RS-II (population range: 0.77–1.40 mmol/l) and 1.12 (0.17) mmol/l in RS-III (population range: 0.79–1.45 mmol/l). Hypophosphatemia was present in 2.7% of the

participants of RS-I, in 3.6% of participants of RS-II and in 2.4% of participants of RS-III. Hyperphosphatemia was present in 1.6% of participants of RS-I, in 1.0% of participants of RS-II and in 2.4% of participants of RS-III.

In UK Biobank, 422,443 participants with an eGFR >60ml/min/1.73m^2 had information on age and serum phosphate. We excluded 9 participants because their serum phosphate concentration was more than 6 standard deviations from the mean. Mean (SD) age was 56.4 (8.1) years. Mean (SD) serum phosphate was 1.16 mmol/l (0.16) in the total population (population range: 0.84–1.47 mmol/l). Hypophosphatemia was present in 1.5% of the total population and hyperphosphatemia in 3.2%. In general, serum phosphate concentrations were higher in UKBB than in RS, which may be explained by that fact that RS blood samples were taken while fasting, while blood sampling in UKBB was performed regardless of fasting status.

Sex difference in the total population

Mean serum phosphate concentrations were higher in women than in men in all RS cohorts and in UKBB (p<0.001) (**Table 1**). The lower limit of the population range for serum phosphate was at least 0.10 mmol/l lower in men than in women in RS and 0.09 mmol/l in UKBB. Similarly, the upper limit of the population range for serum phosphate was at least 0.13 mmol/l higher in women than in men in RS and 0.06 mmol/l in UKBB. Subsequently, hypophosphatemia was more prevalent in men than in women (p<0.001) and hyperphosphatemia was more prevalent in women than in men (p<0.001).

Sex difference per age category

Next, we analyzed the sex difference in serum phosphate concentrations and the prevalence of hypophosphatemia and hyperphosphatemia in different age categories. Mean serum phosphate was significantly different between sexes for all age categories in RS and UKBB (**Table 2**). Moreover, hypophosphatemia was more prevalent in men than in women (T**able 3**) in all age categories in RS and UKBB. Similarly, hyperphosphatemia was more prevalent in women than in men in all age categories older than 50 years in RS and UKBB.

Serum phosphate was significantly different across the different age categories for both sexes (p trend<0.001 for men, p trend <0.001 for women). In RS, men younger than 50 years had higher serum phosphate concentrations than men in the older age categories. Women aged 50-59 years had higher serum phosphate concentrations than women aged 40-49 years (p<0.001) and serum phosphate concentrations decreased in older age categories. UKBB showed similar results.

Table 1. Sex differences in serum phosphate concentrations in RS and UKBB

	Total population	Men	Women	P value
RS-I				
N	3214	1348	1866	
Age (years)	71.5 (6.7)	71.0 (6.2)	71.9 (7.1)	<0.001
Phosphate (mmol/l)	1.11 (0.16)	1.02 (0.14)	1.17 (0.14)	<0.001
P population range (mmol/l)	0.79 – 1.42	0.75 – 1.28	0.89 – 1.46	
Hypophosphatemia	86 (2.7%)	72 (5.6%)	11 (0.6%)	<0.001
Hyperphosphatemia	51 (1.6%)	3 (0.2%)	48 (2.6%)	<0.001
eGFR (ml/min/1.73^m²)	78.0 (9.9)	78.3 (9.9)	77.8 (9.9)	0.175
RS-II				
N	2309	1056	1253	
Age (years)	63.9 (7.2)	63.8 (7.0)	64.0 (7.4)	0.542
Phosphate (mmol/l)	1.08 (0.16)	1.00 (0.14)	1.15 (0.14)	<0.001
P population range (mmol/l)	0.77 – 1.40	0.75 – 1.30	0.85 – 1.43	
Hypophosphatemia	84 (3.6%)	74 (7.0%)	10 (0.8%)	<0.001
Hyperphosphatemia	23 (1.0%)	3 (0.3%)	20 (1.6%)	0.001
eGFR (ml/min/1.73^m²)	83.1 (11.0)	83.3 (10.9)	82.9 (11.1)	0.415
RS-III				
N	3314	1448	1866	
Age (years)	56.7 (6.4)	56.6 (6.1)	56.8 (6.6)	0.284
Phosphate (mmol/l)	1.12 (0.17)	1.04 (0.15)	1.19 (0.15)	<0.001
P population range (mmol/l)	0.79 – 1.45	0.76 – 1.35	0.89 – 1.50	
Hypophosphatemia	86 (2.6%)	71 (4.9%)	15 (0.8%)	<0.001
Hyperphosphatemia	79 (2.4%)	9 (0.6%)	70 (3.8%)	<0.001
eGFR (ml/min/1.73^m²)	87.3 (11.8)	88.0 (11.4)	86.8 (12.0)	0.002
UKBB				
N	422,443	194,989	228,454	
Age (years)	56.4 (8.1)	56.6 (8.2)	56.3 (8.0)	<0.001
Phosphate (mmol/l)	1.16 (0.16)	1.12 (0.16)	1.19 (0.15)	<0.001
P population range (mmol/l)	0.84 – 1.47	0.80 – 1.43	0.89- 1.49	
Hypophosphatemia	6448 (1.5%)	5078 (2.6%)	1370 (0.6%)	<0.001
Hyperphosphatemia	13,429 (3.2%)	3869 (2.0%)	9560 (4.2%)	<0.001
eGFR (ml/min/1.73^m2)	97.4 (14.4)	99.8 (16.3)	95.4 (12.0)	<0.001

Continuous values are displayed as mean (standard deviation), categorical variables are displayed in absolute counts (%). Differences between men and women were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; RS, Rotterdam Study; UKBB, UK Biobank

Next, we calculated the 95% population range of serum phosphate for the total population and for men and women separately. When considering all RS cohorts jointly, 95% of the population had serum phosphate measurements between 0.79 and 1.43

mmol/L. When considering men and women separately, the 95% population range for men was 0.75-1.31 mmol/L and for women 0.88-1.46 mmol/L. The 95% population ranges for the different age categories are depicted in **Table 2**.

Menopausal status

Next, we studied the role of menopausal status on the sex differences in serum phosphate (**Table 4**). Although serum phosphate was significantly higher in both perimenopausal women and postmenopausal women compared to men, postmenopausal women had higher serum phosphate concentrations and higher population ranges than premenopausal women of the same age category.

Sex differences in serum phosphate in impaired kidney function

As a sensitivity analysis, we analyzed sex differences in serum phosphate in participants with impaired kidney function (**Table 5**). Also in this population, mean serum phosphate was higher in women compared to men and hypophosphatemia was less prevalent in women than in men.

Table 2. Sex differences in serum phosphate concentrations and population range in RS and UKBB per age category

	RS-I-II-III					UKBB				
	n	Men	n	Women	p-value	n	Men	n	Women	p-value
40-49yrs	214	1.06 (0.16) 0.72-1.35	273	1.15 (0.16) 0.84 – 1.50	<0.001	45,864	1.13 (0.17) 0.79 – 1.46	54,109	1.15 (0.15) 0.84 – 1.46	<0.001
50-59yrs	1223	1.03 (0.15) 0.75 – 1.33	1551	1.19 (0.15) 0.89 – 1.49	<0.001	62,801	1.12 (0.16) 0.79 – 1.44	78,538	1.21 (0.15) 0.90 – 1.50	<0.001
60-69yrs	1475	1.02 (0.14) 0.75 – 1.30	1836	1.17 (0.14) 0.88 – 1.46	<0.001	85,315	1.11 (0.16) 0.80 – 1.42	93,856	1.21 (0.14) 0.92 – 1.49	<0.001
70-79yrs	777	1.01 (0.14) 0.75 – 1.29	975	1.16 (0.14) 0.88 – 1.45	<0.001	1003	1.11 (0.15) 0.81 – 1.39	950	1.19 (0.15) 0.90 – 1.49	<0.001
>80 yrs	163	1.02 (0.13) 0.77 – 1.26	350	1.15 (0.14) 0.87 – 1.43	<0.001					
P-value for Trend		<0.001		<0.001						

Continuous values are displayed as mean (standard deviation). Differences between men and women were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: RS, Rotterdam Study; UKBB, UK Biobank

Table 3. Sex differences in prevalence of hypophosphatemia in RS and UKBB per age category

	RS-I-II-III					**UKBB**				
	n	Men	n	Women	p-value	n	Men	n	Women	p-value
40-49yrs	214		273			45,864		54,109		
HypoP		10 (4.7%)		4 (1.5%)	0.036		1330 (2.9%)		611 (1.1%)	<0.001
HyperP		2 (0.9%)		9 (3.3%)	0.123		1232 (2.7%)		1535 (2.8%)	0.15
50-59yrs	1223		1551			62,801		78,538		
HypoP		75 (6.1%)		12 (0.8%)	<0.001		1690 (2.7%)		417 (0.5%)	<0.001
HyperP		7 (0.6%)		56 (3.6%)	<0.001		1277 (2.0%)		3889 (5.0%)	<0.001
60-69yrs	1475		1836			85,315		93,856		
HypoP		83 (5.6%)		6 (0.3%)	<0.001		2037 (2.4%)		338 (0.4%)	<0.001
HyperP		5 (0.3%)		46 (2.5%)	<0.001		1350 (1.6%)		4104 (4.4%)	<0.001
70-79yrs	777		975			1003		950		
HypoP		46 (5.9%)		11 (1.1%)	<0.001		21 (2.1%)		4 (0.4%)	0.001
HyperP		1 (0.1%)		21 (2.2%)	<0.001		10 (1.0%)		32 (3.4%)	<0.001
>80 yrs	163		350							
HypoP		6 (3.7%)		3 (0.9%)	0.023	-	-	-	-	-
HyperP		0		6 (1.7%)	-	-	-	-	-	-

Categorical values are displayed in absolute counts (%). Differences between men and women were analyzed using $\chi 2$ test for categorical variables. Abbreviations: RS, Rotterdam Study; UKBB, UK Biobank

Table 4. Differences in mean serum phosphate in men, perimenopausal women and postmenopausal women in RS and UKBB

	n	Men	n	Perimenopausal women	n	Postmenopausal women	p-value[a]	p-value[b]	p-value[c]
RS-I-II-III									
40-49yrs	214	1.06 (0.16) 0.72-1.35	205	1.13 (0.15) 0.86 – 1.50	67	1.20 (0.17) 0.77 – 1.56	<0.001	<0.001	0.001
50-59yrs	1223	1.03 (0.16) 0.75 – 1.33	311	1.15 (0.16) 0.82 – 1.47	1235	1.20 (0.15) 0.91 – 1.50	<0.001	<0.001	<0.001
UKBB									
40-49yrs	45,864	1.13 (0.17) 0.79 – 1.46	42,630	1.15 (0.15) 0.85 – 1.45	4279	1.21 (0.16) 0.89 – 1.52	<0.001	<0.001	<0.001
50-59yrs	62,801	1.12 (0.16) 0.79 – 1.44	10,460	1.16 (0.15) 0.85 – 1.45	52,734	1.22 (0.15) 0.92 – 1.51	<0.001	<0.001	<0.001

Continuous values are displayed as mean (standard deviation). [a] student t-test comparing men and perimenopausal women. [b] student t-test comparing men and postmenopausal women. [C]student t-test comparing perimenopausal and postmenopausal women. Abbreviations: RS, Rotterdam Study; UKBB, UK Biobank

Table 5. Differences in serum phosphate in participants with impaired kidney function, stratified by sex.

	Men	Women	p-value
RS-I-II-III			
N	392	576	
Age, years	74.9 (8.5)	76.4 (9.2)	0.011
Phosphate, mmol	1.04 (0.15)	1.15 (0.14)	<0.001
P population range	0.78 – 1.34	0.88 – 1.44	
HypoP	15 (3.8%)	3 (0.5%)	<0.001
HyperP	4 (1.0%)	11 (1.9%)	0.271
eGFR	49.5 (9.9)	51.0 (7.9)	0.015
UKBB			
N	3155	3490	
Age, years	62.9 (6.1)	62.4 (6.0)	<0.001
Phosphate, mmol/L	1.15 (0.19)	1.22 (0.18)	<0.001
P population range	0.80 – 1.56	0.90 – 1.59	
HypoP	83 (2.6%)	23 (0.7%)	<0.001
HyperP	161 (5.1%)	292 (8.4%)	<0.001
eGFR	49.6 (11.0)	51.2 (9.4)	<0.001

Continuous values are displayed as mean (standard deviation)), categorical variables are displayed in absolute counts (%). Differences between men and women were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; HypoP, hypophosphatemia; HyperP, hyperphosphatemia; RS, Rotterdam Study; UKBB, UK Biobank

DISCUSSION

Our analyses in two population-based studies consistently showed that women have higher serum phosphate levels than men. The difference in serum phosphate between men and women becomes more pronounced after menopause. Moreover, while in both elderly men and women serum phosphate concentrations decrease with age, women appear to go through an initial increase in serum phosphate around menopause. Moreover, the population range for serum phosphate is higher in women than in men resulting in a higher prevalence of hypophosphatemia in men and a higher prevalence of hyperphosphatemia in women.

Our findings are in line with previous studies by de Boer *et al.* and Cirillo *et al*.[13,18] Cirillo *et al.* conducted a study in 2107 men and 2560 women ranging 18 to 97 years and found that fasting serum phosphate concentrations decreased with age in both sexes and were similar in men and women younger than 45 years. Interestingly, women showed an increase in serum phosphate concentrations around the age of 45 years. Moreover, the tubular maximum reabsorption rate of phosphate (TmP/GFR) displayed a similar pattern: a decrease with age in both sexes with an transient increase in women around the age of 45 years.[13] This suggests that the differences in serum phosphate concentrations

between men and women are the result of altered renal phosphate handling. A similar trend in serum phosphate was observed in a hospital information system by Koek *et al*, albeit this study included subjects that were either visiting an out-patient clinic or were admitted to the hospital and the fasting status was unknown[14].

Our results confirm that there is a sexual dimorphism in phosphate homeostasis. The fact that postmenopausal women have higher serum phosphate concentrations than premenopausal women suggests that the hormonal changes during menopause play a role. Indeed, it has been shown that estrogen treatment induces renal phosphate wasting and hypophosphatemia in rats due to downregulation of the sodium-phosphate transporters in the proximal tubule[19]. Moreover, Dick et al. reported a negative association of free serum estradiol with renal tubular phosphate reabsorption in postmenopausal women and Meng et al. reported a negative association of total estradiol and testosterone with serum phosphate in a cohort of community-living older men[20,21]. Previous research conducted in RS by our group showed that both serum testosterone and estradiol influence serum phosphate in both sexes but that only serum testosterone may explain the sex differences in serum phosphate[22].

An increasing body of evidence suggest that increased, yet normal, serum phosphate is associated with an increased risk for mortality from COPD, cardiovascular disease and coronary artery calcification (CAC) in the general population[9-12]. Importantly, some of these associations show marked sex-differences and are seen mainly in men. Our group found that serum phosphate is associated with CAC in the general population with stronger effects in men[10]. Moreover, we found that the association between serum phosphate and CAC became apparent at lower serum phosphate concentrations in men than in women, suggesting that the deleterious effects of serum phosphate occur at lower serum phosphate concentrations in men than in women[10]. These sex-specific findings strengthen the relevance for accurate reference values. In the current study, we found that the population range for serum phosphate in the general population is lower in men than in women. Taken together, these findings warrant sex-specific reference ranges. Remarkably, age- and sex- related reference ranges already exist for the TmP/GFR[23]. Based on our findings, we propose to introduce sex-specific reference ranges for serum phosphate e.g., 0.75 – 1.35 mmol/L (2.3 – 4.2 mg/dL) in males and 0.85 – 1.45 mmol/L (2.6 – 4.5 mg/dL) in women older than 45 years. We consider that this first effort in correcting the phosphate references ranges for sex has important implications for public health.

This study has several limitations. The population of both RS and UKBB are composed mainly of European Caucasians, which refrains us from drawing conclusion on other populations or ethnic groups. Moreover, blood samples were taken regardless of fasting status in UKBB and intake influences serum phosphate concentrations[24]. For this

reason, we calculated the population ranges for serum phosphate in RS only. Still, we found that women had higher serum phosphate concentrations than men also in UKBB suggesting that sex-differences are present irrespective of fasting status. In addition, RS and UKBB is composed of participants aged 40 years and older, which refrains us from drawing conclusions on sex-differences in younger people. Future studies should include participants younger than 40 years.

Our study has several strengths. To the best of our knowledge, this is the largest study conducted on age and sex related differences in serum phosphate concentrations to date. Due to our large sample size, we were able to analyze serum phosphate in different age categories and to study the role of menopause. Our findings are important for several reasons: future association analyses with serum phosphate should take in account sex and age; laboratories of health care institutions should consider sex-specific reference ranges; clinicians who treat patients with phosphate disturbances should be aware of sex-differences in serum phosphate, which could also prevent unnecessary investigations into decreased or increased serum phosphate concentrations.

In conclusion, this study in two population-based cohort studies showed that women have higher serum phosphate concentrations than men and that women show a marked increase in serum phosphate during menopause. Moreover, the population range for serum phosphate was higher in women than in men. These findings have important clinical consequences. Given the sex-differences in the associations of serum phosphate with morbidity and mortality, sex-specific references ranges for serum phosphate are warranted. We propose to introduce sex-specific reference ranges for serum phosphate e.g., 0.75 – 1.35 mmol/L in males and 0.85 – 1.45 mmol/L in women older than 45 years. We suggest to restudy the concept of phosphotoxicity with these new values, according to sex.

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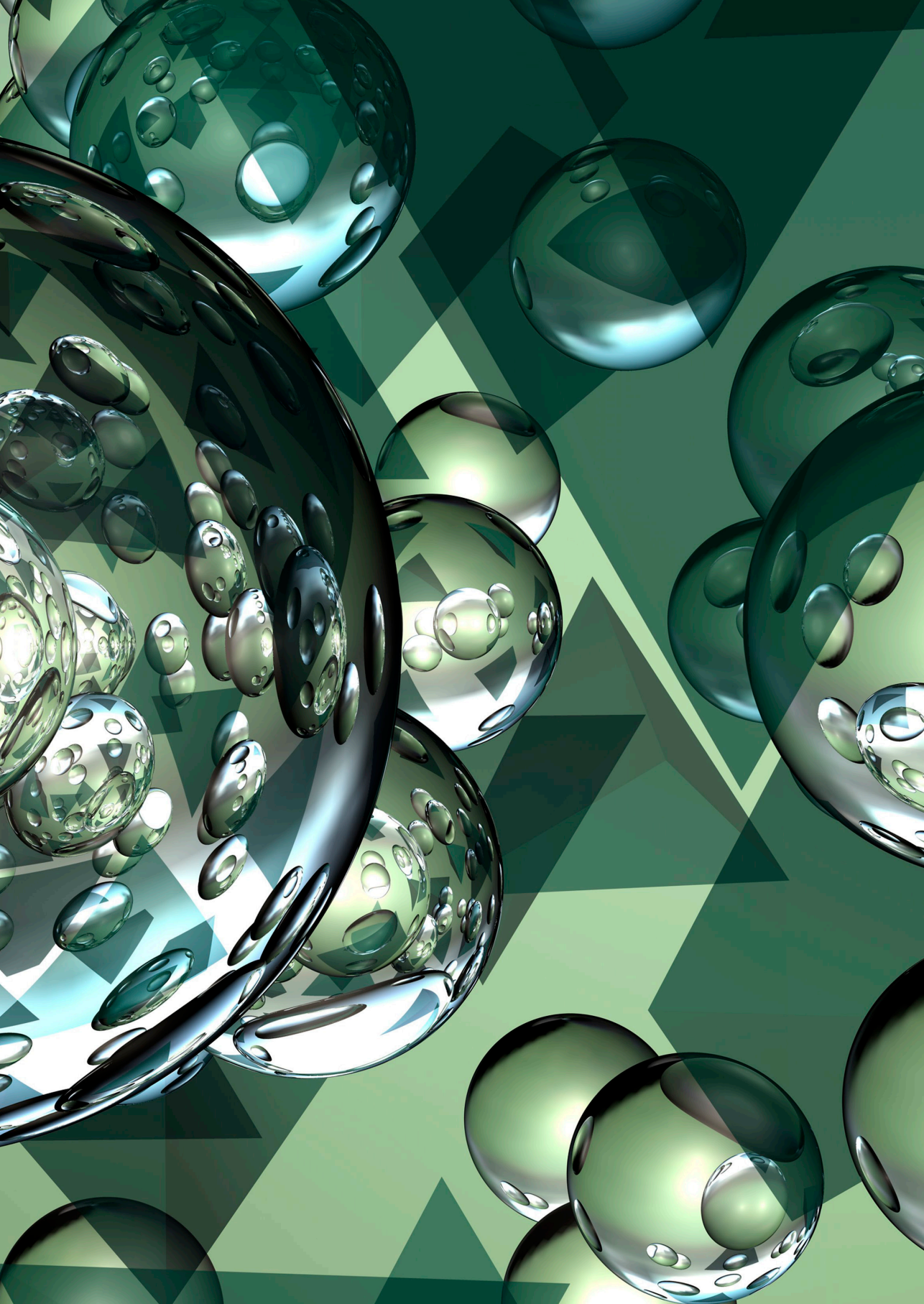

Sexual dimorphisms in serum calcium and phosphate concentrations in the Rotterdam Study

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ABSTRACT

Sex differences in serum phosphate and calcium have been reported but the exact nature and underlying regulatory mechanisms remain unclear. We aimed to compare calcium and phosphate concentrations between sexes, and explore potential covariates to elucidate underlying mechanisms of sex differences in a prospective, population-based cohort study. Pooled data of subjects > 45 years from three independent cohorts of the Rotterdam Study (RS) were used: RS-I-3 (n=3623), RS-II-1 (n=2394), RS-III-1 (n=3241), with separate analyses from an additional time point of the first cohort RS-I-1 (n=2688). Compared to men, women had significantly higher total serum calcium and phosphate concentrations which was not explained by BMI, kidney function nor smoking. Adjustment for serum estradiol diminished sex differences in serum calcium while adjustment for serum testosterone diminished sex differences in serum phosphate. Adjustment for vitamin D and alkaline phosphatase did not change the association between sex and calcium or phosphate in RS-I-1. In the sex-combined group, both serum calcium and phosphate decreased with age with a significant interaction for sex differences for serum calcium but not phosphate. In sex-stratified analyses, serum estradiol but not testosterone was inversely associated with serum calcium in both sexes. Serum estradiol was inversely associated with serum phosphate in both sexes to a similar degree, while serum testosterone was inversely associated with serum phosphate in both sexes with an apparent stronger effect in men than in women. Premenopausal women had lower serum phosphate compared to postmenopausal women. Serum testosterone was inversely associated with serum phosphate in postmenopausal women only. In conclusion, women > 45 years have higher serum calcium and phosphate concentrations compared to men of similar age, not explained by vitamin D or alkaline phosphatase concentrations. Serum estradiol but not testosterone was inversely associated with serum calcium while serum testosterone was inversely associated with serum phosphate in both sexes. Serum testosterone may in part explain sex differences in serum phosphate while estradiol could partly explain sex differences in serum calcium.

INTRODUCTION

Calcium and phosphate are important electrolytes in human physiology. Calcium is one of the most abundant cations in the body. It is crucial for several metabolic processes such as neural transmission, blood coagulation and cell proliferation, and pivotal for bone mineralization. Approximately 51% of total calcium is bound to proteins such as albumin and globulin while the remainder circulates in ionic form (free serum calcium or Ca2+)[1]. Serum calcium concentrations are tightly controlled through the interaction of the intestines, kidneys, bone, and parathyroid glands. Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) are the major hormones controlling calcium homeostasis by stimulating calcium absorption in the intestines, calcium reabsorption in the kidney and bone resorption.

Phosphate is an important electrolyte in energy metabolism and is part of DNA and RNA structures[2,3]. Moreover, it is incorporated in extracellular matrix as hydroxyapatite during bone formation. Serum calcium and phosphate concentrations influence each other[1,2]. Serum phosphate is predominantly controlled through urinary excretion by the actions of PTH and the osteocyte-derived hormone fibroblast growth factor 23 (FGF23). Additionally, $1,25(OH)_2D_3$ regulates phosphate absorption from the diet in the intestine[2]. Phosphate is present in serum mainly in a free and ultra-filterable form (85-90%), whilst the remainder (10-15%) is bound to proteins[3,4].

Serum calcium and phosphate imbalance has been linked to several disorders such as cardiovascular disease, COPD, metabolic syndrome and osteoporosis, and mortality[4-9]. Furthermore, the calcium-phosphate product is associated with morbidity and mortality in patients with end-stage renal disease[7], although the recent KDIGO guideline suggests no additional value of this construct over individual serum concentrations of calcium and phosphate in patients with chronic kidney disease (CKD)[10]. Some of these disorders display marked sex-specific incidences: the incidence of cardiovascular diseases has been found to be lower in women compared to men[5,11]. Moreover, Tohno *et al*. found sex differences in the accumulation of calcium and phosphate in coronary arteries[12]. Thus, it is possible that sex differences in the incidence of these disorders might be related to calcium and phosphate homeostasis and that the underlying mechanisms may have clinical consequences.

There have been several studies evaluating sex differences in serum calcium and phosphate. Although these studies found sex differences between post-menopausal women and men, the findings were less consistent for serum calcium than for serum phosphate[4,13-15]. Studies evaluating total serum calcium concentrations have found differences between pre- and postmenopausal women, and between men and women

at various ages, but these results were either inconsistent or sex differences were not systematically investigated in different age groups: Lindgarde *et al*. and Roof *et al*. reported that serum total calcium was significantly higher in young men compared to women, while Håglin *et al*. found that women had higher serum calcium concentrations compared to men[13,16-19]. More consistent data are available showing that postmenopausal women have higher serum phosphate concentrations compared to men of similar age [5,18,20-23]. In a previous study conducted in three different samples extracted from a hospital information system, we found that, above 45 years of age, women have higher calcium and phosphate concentrations than men[24].

In this study, we compared serum calcium and phosphate concentrations between men and women in a population-based cohort study of elderly white Caucasians, i.e., the Rotterdam Study, and explored the role of potential confounders including age, vitamin D and sex hormones and how they influence calcium and phosphate concentrations in both sexes.

METHODS

Rotterdam Study

The Rotterdam Study (RS) is a large prospective population-based cohort study of Caucasian subjects aged 45 years and older, living in the Ommoord district of Rotterdam, The Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases in the elderly. Rationale and design have been described previously [25]. It is now composed of four cohorts. The first cohort, named RS-I, initiated in 1989. RS-II, RS-III and RS-IV followed in 2000, 2005 and 2017, respectively. Participants have been followed through several visits since recruitment, which are all similar in design and data collection.

Serum phosphate and calcium concentrations have been measured in the baseline visit of RS-I, named RS-I-1; in the second follow up visit of RS-I, named RS-I-3; and in the baseline visits of RS-II and RS-III. The participants in RS-I and RS-II are all aged 55 years and older, while RS-III consists of participants aged 45 years and older. Serum creatinine, 25-hydroxyvitamin D (25(OH)D) and estradiol and testosterone concentrations were measured in all cohorts. All measurements were taken in a fasting state, except for RS-I-1. A total of 3623 participants from RS-I-3, 2394 from RS-II and 3241 from RS-III with complete information on fasting serum calcium, serum phosphate and covariates were included in the main analyses of this study.

A total of 2688 participants from RS-I-1 had information on non-fasting serum calcium,

phosphate and covariates. In addition, serum albumin, alkaline phosphatase (ALP) and $1,25(OH)_2D_3$ concentrations were measured only in RS-I-1.

Assay Methods

Serum samples from subjects were analyzed directly after sample collection at the Department of Internal Medicine of the Erasmus MC Rotterdam, The Netherlands,.
Serum total calcium, inorganic phosphate, albumin and ALP were measured using the Hitachi 917 Analyzer (Roche, Mannheim, Germany). The corresponding inter-assay coefficients of variation (CV) were <0.7% for serum calcium and phosphate. The calcium-phosphate product was calculated by multiplying the subjects' total serum calcium and phosphate concentrations, and expressed in $mmol^2/L^2$. Since Payne reported in 1973 that serum albumin influences serum calcium concentrations, we calculated corrected serum calcium concentrations using the formula: calcium (corrected) = calcium measured + 0.02 (42 - albumin measured) in RS-I-1 [26].

In RS-I-3, RS-II-1 and RS-III-1, serum 25(OH)D concentrations were measured using electrochemiluminescence immunoassays (COBAS < Roche Diagnostics GmbH, Germany). The inter-assay CV for 25(OH)D was <7.8%. For RS-I-1, serum $1,25(OH)_2D_3$ and 25(OH)D concentrations were measured using 125I-radioimmunoassays (RIA) with inter-assay CVs of 7-11% and 7.9% (from IDS, Boldon, UK and DiaSorin, Stillwater, MN, USA; respectively)[27,28].

Total estradiol concentrations were determined using a COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH) with a lower limit of detection of 18.4 pmol/L. Total testosterone concentrations were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The inter-assay CVs were <4.6% for serum estradiol and <5.7% for serum testosterone[29].

Due to limited amount of plasma per subject in RS-I-1, not all hormone concentrations could be determined in all subjects.

Assessment of covariates

BMI was calculated as the body mass divided by the square of the body height, measured in standing position without shoes, and expressed in kg/m^2. eGFR ($mL/min/1.73\ m^2$) was estimated using the Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine concentrations[30,31]. In all cohorts, smoking status was assessed during home interviews. Women were interviewed for having previously used hormone replacement therapy (HRT). No data on current use of HRT was documented. In RS-II-1 and RS-III-1, women were interviewed for their current menopausal status. Women were considered perimenopausal if they were within one year of their last menstruation.

Statistical Analyses

Data from RS-I-3, RS-II-1 and RS-III-1 were pooled (and from now on called "the pooled dataset") in order to evaluate overall sex differences for serum calcium and phosphate. RS-I-I was analyzed separately because of non-fasting condition and availability of additional covariates. Differences between men and women were tested with independent t-tests for continuous variables and chi-square tests for categorical variables. Multivariate linear regression models were used to study sex differences in serum calcium and phosphate. All analyses were performed using Z-scores for the biomarkers, calculated by subtracting the mean from the value divided by the standard deviation. The analyses included covariates that are known to differ between the sexes and covariates that potentially influence the outcome variables serum calcium and phosphate: age, BMI, smoking and eGFR. Analyses were additionally adjusted for cohort to correct for unexpected categorical effects, with RS-I-3 being given dummy variables coded 0-0-1, RS-II-1 given dummy variables coded 0-1-0, and RS-III-1 given dummy variable 1-0-0. Analyses were performed in the total population and sex-stratified. In the total population, we included interaction terms of age with sex to explore whether sex differences in serum calcium and phosphate vary according to age. In order to evaluate the influence of sex hormones and 25(OH)D on sex differences in serum calcium and phosphate concentrations, we tested the change in the beta-coefficient of observed sex differences in a multivariate linear regression model in the total population. A change of > 20% in the beta-coefficient of sex, after correction for the specific variable, indicated that the variable has an important influence on the observed sex differences. Because sex hormone concentrations are associated with sex we tested for multicollinearity. A variance inflation factor (VIF) of 10 was applied as a threshold for multicollinearity[32].

To evaluate age in relation with serum calcium and phosphate, subjects in the pooled dataset were stratified into consecutive decades and stratified by sex. Age-group differences were tested per sex using ANCOVA in a general linear model and adjusted for BMI, smoking, eGFR and cohort.

Sex stratified two-tailed partial correlation analyses in the pooled dataset were performed to test for correlations between serum 25(OH)D, estradiol and testosterone and serum calcium and phosphate. In order to study the influence of sex hormones and 25(OH)D on serum calcium and phosphate in men and women separately, we performed sex-stratified multivariate regression analyses adjusting for serum estradiol, testosterone and 25(OH)D. Analyses in women were additionally adjusted for previous hormone replacement therapy (HRT) and menopausal status. Furthermore, analyses were performed in pre- and postmenopausal women separately. Lastly, we compared beta-coefficients for age and serum calcium or phosphate with and without addition of serum estradiol, testosterone and 25(OH)D. All results are expressed per 1-SD increase

of serum estradiol, testosterone or 25(OH)D, unless otherwise stated.

Sensitivity analyses

All analyses were also performed in RS-I-1 and were further adjusted for serum 25(OH)D, $1,25(OH)_2D_3$ and ALP. Data on sex hormones as well as 25(OH)D and $1,25(OH)_2D_3$ were only available in a subset of RS-I-1 (**Supplementary table 8)**. Because a large proportion of serum calcium is bound to albumin, sex differences in serum albumin concentrations were analyzed and serum calcium concentrations were corrected for serum albumin in RS-I-1. Furthermore, analyses were repeated in the subset of RS-I-1 with albumin concentrations within a narrow range (38-42 g/L).

Statistical analyses were performed using SPSS (version 23). Due to the explorative nature of the study, statistical significance of two-sided tests was defined as $p < 0.05$.

Ethical approval

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. All methods were carried out in accordance with relevant guidelines and regulations.

RESULTS

The general characteristics of the pooled dataset are described in **Table 1**. The general characteristics of the individual cohorts are described in **Supplemental Table 1 to 3.** Women had higher serum calcium and phosphate concentrations compared to men (mean (SD) serum calcium: 2.42 (0.10) mmol/L in men, 2.44 (0.10) mmol/L in women, $p<0.001$; mean(SD) serum phosphate: 1.02 (0.15) mmol/L in men, 1.17(0.15) mmol/L in women, $p<0.001$). Consequently, the calcium-phosphate product was higher in women compared to men. Serum 25(OH)D, estradiol and testosterone concentrations were significantly lower in women compared to men. The majority of women was postmenopausal.

Table 1. Baseline characteristics of the pooled dataset

	Men	Women	*P-value*
N	4043	5215	
Age (years)	64.5 (9.4)	65.3 (9.8)	<0.001
Calcium (mmol/L)	2.42 (0.10)	2.44 (0.10)	<0.001
Phosphate (mmol/L)	1.02 (0.15)	1.17 (0.15)	<0.001
Calcium-phosphate product ($mmol^2/L^2$)	2.47 (0.39)	2.86 (0.39)	<0.001
25(OH)D (nmol/L)	59.9 (27.9)	53.5 (27.2)	<0.001
eGFR ($mL/min/1.73\ m^2$)	80.0 (16.2)	78.7 (15.9)	<0.001
Estradiol (pmol/L)	104.2 (40.7)	64.5 (102.5)	<0.001
Testosterone (nmol/L)	17.17 (5.96)	0.94 (0.81)	<0.001
BMI (kg/m^2)	27.0 (3.6)	27.5 (4.6)	<0.001
Current smoking, n(%)	805 (19.9%)	992 (19.0%)	0.384
Ever HRT use, n(%)		847 (18.2%)	
Postmenopausal, n(%)	-	4802 (92.1%)	

Continuous values are displayed as mean (standard deviation)), categorical variables are displayed in absolute counts (%). Differences between males and females were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; BMI, body mass index; eGFR, estimated glomerular filtration rate; HRT, hormone replacement therapy; ND, not determined; RS, Rotterdam Study.

Sex differences in serum calcium and serum phosphate

In multivariate linear regression analyses in the total population, we found a significant association of sex on serum calcium and phosphate (serum calcium: β: 0.274, 95%CI: 0.234 to 0.314; P < 0.001; serum phosphate: β: 0.917, 95%CI: 0.881 to 0.953; P < 0.001). Moreover, age was inversely associated with both serum calcium and serum phosphate concentrations (serum calcium: β: -0.120, 95%CI: -0.152 to -0.088; P < 0.001; serum phosphate: β:-0.038, 95%CI:-0.067 to-0.009; P < 0.001) (**Table 2**).

Interaction analysis of age and sex showed an interaction for age and sex on serum calcium concentrations with a standardized beta coefficient of 0.43 *(P* <0.001) for the interaction term age*sex. For analyses with serum phosphate modelled as the outcome, the interaction term age*sex was not significant *(P* = 0.3). Next, we analyzed the influence of serum testosterone, estradiol and 25(OH)D concentrations on sex differences in serum calcium and phosphate concentrations in the total population (**Table 3**).

Table 2. Association between sex and age, and serum calcium and phosphate concentrations in the pooled dataset

		Serum calcium (mmol/L)		Serum phosphate (mmol/L)	
Pooled dataset N=9258		Standardized Beta-coefficient (95% CI)	P value	Standardized Beta-coefficient (95% CI)	P value
	Sex	0.274 (0.234 to 0.314)	<0.001	0.917 (0.881 to 0.953)	<0.001
	Age	-0.120 (-0.152 to-0.088)	<0.001	-0.038 (-0.067 to-0.009)	0.011

βs were obtained from linear regression models. All models were adjusted for sex, age in decades, cohort, BMI, eGFR and smoking

Table 3. Influence of serum testosterone, estradiol and 25(OH)D on sex differences in serum calcium and phosphate concentrations in the pooled dataset

		Serum calcium (mmol/l)		Serum phosphate (mmol/l)	
Pooled dataset N=9270		Standardized Beta-coefficient sex (95% CI)	% change beta-coefficient from model 1	Standardized Beta-coefficient sex (95% CI)	% change beta-coefficient from model 1
	Model 1[a]	0.274 (0.234 to 0.314)		0.917 (0.881 to 0.953)	
	Model 2[b]	0.218 (0.177 to 0.259)	-20.4%	0.856 (0.819 to 0.893)	-6.7%
	Model 3[c]	0.364 (0.274 to 0.455)	32.8%	0.572 (0.490 to 0.654)	-37.6%
	Model 4[d]	0.351 (0.261 to 0.441)	28.1%	0.559 (0.478 to 0.640)	-39.0%
	Model 5[e]	0.284 (0.244 to 0.3240	3.6%	0.915 (0.879 to 0.951)	-0.2%

βs were obtained from linear regression models and expressed per 1-SD increase in serum estradiol, testosterone or 25(OH)D. [a]Model 1: adjusted for age in decades, cohort, BMI, eGFR and smoking. [b]Model 2: model 1 and estradiol. [c]Model 3: model 1 and testosterone. [d]Model 4: model 1 and estradiol and testosterone. [e]Model 5: model 1 and 25(OH)D.

Multicollinearity statistics showed a VIF of 1.068 for serum estradiol and 5.077 for serum testosterone. Serum estradiol diminished the effect size for the association between sex and serum calcium by 20.4% (β: 0.218, 95%CI: 0.177 to 0.259; P < 0.001), while serum testosterone enhanced the effect size by 32.8%. The effect size for the association between sex and serum phosphate did not change after adjusting for serum estradiol. Adjusting for serum testosterone diminished the effect size of the association between sex and serum phosphate by 37.6% but it remained significant (β: 0.559, 95%CI: 0.478 to 0.640; P < 0.001). Serum 25(OH)D did not change the effect size for the associations between either sex and serum calcium or sex and serum phosphate.

After stratification of subjects into age in decades, serum calcium and phosphate showed a relation with age in both men and women, with calcium and phosphate levels being lower in each consecutive decade (**Supplementary Table 4**).

In sex-stratified correlation analyses, a negative correlation was found between serum estradiol and serum calcium in both sexes (men: ρ: -0.110, P <0.001; women: ρ: -0.121, P <0.001), but not between testosterone and serum calcium. Serum 25(OH)D concentrations were positively correlated with serum calcium in both sexes (men: ρ: 0.040, P =0.01; women: ρ: 0.070, P <0.001). Both serum estradiol and testosterone were negatively correlated with serum phosphate in both sexes (for serum estradiol: men: ρ: -0.122, P <0.001; women: ρ:-0.142, P <0.001; for serum testosterone: men: ρ:-0.118, P <0.001; women: ρ:-0.046, P <0.001) (**Table 4**). Serum 25(OH)D concentrations were negatively correlated with serum phosphate in men only. In correlation analysis stratified by menopausal status, serum estradiol was negatively correlated with serum calcium and serum phosphate in both pre- and postmenopausal women. Serum testosterone was not correlated with serum calcium in both pre- and postmenopausal women, but it was negatively correlated with serum phosphate in postmenopausal women (ρ:-0.049, P <0.001). Serum 25(OH)D concentrations were positively correlated with serum calcium but not with serum phosphate in both pre- and postmenopausal women.

Table 4 Partial correlation analysis in the pooled data set of RS-I-3, RS-II-1 and RS-III-1 for serum calcium and phosphate with 25(OH)D, estradiol and testosterone, stratified by sex and menopausal status.

		Serum calcium		Serum phosphate	
		R	p value*	R	p value*
By sex					
Men	Estradiol	-0.110	<0.001	-0.122	<0.001
	Testosterone	0.028	0.080	-0.118	<0.001
	25(OH)D	0.040	0.010	-0.039	0.014
Women	Estradiol	-0.121	<0.001	-0.142	<0.001
	Testosterone	0.004	0.785	-0.046	<0.001
	25(OH)D	0.070	<0.001	0.016	0.248
By menopausal status					
Premenopausal women	Estradiol	-0.109	0.045	-0.127	0.019
	Testosterone	0.046	0.397	0.052	0.338
	25(OH)D	0.080	0.138	0.011	0.845
Postmenopausal women	Estradiol	-0.136	<0.001	-0.155	<0.001
	Testosterone	0.003	0.858	-0.049	<0.001
	25(OH)D	0.067	<0.001	0.015	0.300

*Analyses are adjusted for age, BMI, eGFR, smoking and cohort (and menopausal status and HRT use in women)

Sex-stratified multivariate linear regression analyses revealed an inverse association of age on serum calcium in both sexes, while age was inversely associated with serum phosphate in women only (**Table 5)**. Adding serum testosterone to the model revealed that serum testosterone was not associated with serum calcium in both sexes, but it

was inversely associated with serum phosphate in both sexes (men: β: -0.123, 95%CI: -0.155 to-0.091; P < 0.001; women: β:-0.047, 95%CI:-0.073 to-0.021; P < 0.001). Serum estradiol was inversely associated with serum calcium and serum phosphate in both sexes. Serum 25(OH)D was positively associated with serum calcium in both sexes and negatively associated with serum phosphate in men only. Serum testosterone, estradiol and 25(OH)D did not influence the effect size for age on serum calcium in men. **(Supplementary Table 5**).

Table 5. Sex-stratified beta-coefficients for serum calcium and phosphate levels, according to covariates, in the pooled dataset

		Serum calcium (mmol/L)		**Serum phosphate (mmol/L)**	
Men (n=4043)		Standardized Beta-coefficient (95% CI)	P value	Standardized Beta-coefficient (95% CI)	P value
	Age (per decade)	-0.166 (-0.217 to-0.116)	<0.001	-0.047 (-0.098 to 0.005)	0.075
	Testosterone	0.028 (-0.003 to-0.060)	0.081	-0.123 (-0.155 to-0.091)	<0.001
	Estradiol	-0.113 (-0.146 to-0.081)	<0.001	-0.132 (-0.165 to-0.098)	<0.001
	25(OH)D	0.040 (0.009 to 0.070)	0.011	-0.039 (-0.070 to-0.008)	0.013
Women (n=5215)		Standardized Beta-coefficient (95% CI)	P value	Standardized Beta-coefficient (95% CI)	P value
	Age (per decade)	-0.119 (-0.163 to-0.075)	<0.001	-0.081 (-0.124 to-0.038)	<0.001
	Testosterone	0.004 (-0.023 to 0.031)	0.774	-0.047 (-0.073 to-0.021)	<0.001
	Estradiol	-0.104 (-0.134 to-0.073)	<0.001	-0.131 (-0.160 to-0.101)	<0.001
	25(OH)D	0.077 (0.048 to 0.107)	<0.001	0.014 (-0.015 to 0.043)	0.344

βs were obtained from linear regression models and expressed per 1-SD increase in serum estradiol, testosterone or 25(OH)D. All models were adjusted for age, cohort, BMI, eGFR and smoking (and menopausal status and previous HRT use in women).

Analyses in women were further stratified by menopausal status (**Table 6, Supplementary Table 6**). Postmenopausal women had higher serum phosphate and testosterone concentrations and higher calcium-phosphate product levels than premenopausal women. Serum 25(OH)D and estradiol was lower in postmenopausal women. Age was positively associated with serum calcium in premenopausal women and inversely associated with serum calcium and serum phosphate in postmenopausal women. Serum testosterone was not significantly associated with serum calcium nor serum phosphate in premenopausal women but there was a significant inverse association between serum

testosterone and phosphate in postmenopausal women (β: -0.048, 95%CI: -0.075 to -0.020; P < 0.001). Estradiol was inversely associated with serum calcium and phosphate in postmenopausal women but in premenopausal women only with serum phosphate and not serum calcium. Serum 25(OH)D was positively associated with serum calcium in both pre- and postmenopausal women but not with serum phosphate.

Table 6. Beta-coefficients, stratified by menopausal status, for serum calcium and phosphate levels, according to covariates, in the pooled dataset

		Serum calcium (mmol/L)		**Serum phosphate (mmol/L)**	
Premenopausal (n=413)		Standardized Beta-coefficient (95% CI)	P value	Standardized Beta-coefficient (95% CI)	P value
	Age (per decade)	0.360 (0.022 to 0.699)	0.037	0.308 (-0.026 to 0.642)	0.070
	Testosterone	0.038 (-0.060 to 0.137)	0.448	-0.027 (-0.124 to 0.070)	0.587
	Estradiol	-0.072 (-0.169 to 0.025)	0.143	-0.099 (-0.195 to-0.004)	0.041
	25(OH)D	0.102 (0.001 to 0.203)	0.048	-0.013 (-0.114 to 0.087)	0.793
Postmenopausal (n=4802)		Unstandardized Beta-coefficient age (95% CI)	P value	Unstandardized Beta-coefficient age (95% CI)	P value
	Age (per decade)	-0.129 (-0.173 to-0.084)	<0.001	-0.087 (-0.131 to-0.043)	<0.001
	Testosterone	0.003 (-0.025 to 0.031)	0.843	-0.048 (-0.075 to-0.020)	<0.001
	Estradiol	-0.128 (-0.157 to-0.099)	<0.001	-0.162 (-0.190 to-0.134)	<0.001

βs were obtained from linear regression models and expressed per 1-SD increase in serum estradiol, testosterone or 25(OH)D. All models were adjusted for age, cohort, BMI, eGFR and smoking (and previous HRT use in postmenopausal women).

Sensitivity analyses

The baseline characteristics of RS-I-1 are depicted in **Supplementary Table 8**. Non-fasting serum calcium and phosphate concentrations were higher in women than in men (mean (SD) serum calcium: 2.36 (0.14) mmol/L in men, 2.37 (0.13) mmol/L in women, p=0.002; mean (SD) serum phosphate: 1.09 (0.19) mmol/L in men, 1.23 (0.17) mmol/L in women, 0<0.001). Concentrations of $1,25(OH)_2D_3$ were not significantly different between the sexes. ALP concentrations were higher in women compared to men (p <0.001). Serum 25(OH)D, testosterone and estradiol concentrations were lower in women than in men. Multivariate linear regression analysis showed a significant positive association of sex on serum calcium and serum phosphate. Age was negatively associated with serum phosphate. The association between age and serum calcium did not reach significance

(**Supplementary Table 9)**. The effect size of the association between sex and serum calcium and phosphate did not change after adjusting for serum 25(OH)D, serum $1.25(OH)_2D_3$, serum 25(OH)D and $1.25(OH)_2D_3$, or ALP (**Supplementary Table 10**)

Lastly, we evaluated sex differences in serum albumin concentrations. Serum albumin concentrations were not significantly different between men and women. After correcting serum calcium for serum albumin concentrations according to the aforementioned formula, the sex differences in serum calcium concentrations remained, with women having higher corrected serum calcium concentrations compared to men (data not shown).

To exclude an impact of high or low albumin concentrations on calcium concentrations, we performed sensitivity analyses, restricting our data to only those subjects with albumin concentrations within a narrow range (38-42 g/L). In this subset, accounting for 42% of the total RS-I-1 dataset, we found similar results as in the overall data (mean (SD) serum calcium: 2.34 (0.13) mmol/L in men, 2.36 (0.13) mmol/L in women, p=0.04)

DISCUSSION

In this study, sexual dimorphism in serum calcium and phosphate concentrations and the role of potential confounders were investigated. In a pooled analysis of subjects aged 45 years and older in three cohorts from the Rotterdam Study, we showed that serum calcium, phosphate and the calcium-phosphate product were significantly higher in women compared to men. Both serum calcium concentrations, in a sex-dependent manner, and serum phosphate concentrations, in a sex-independent manner, declined with aging. There are a few older and mainly small-sized studies that have found higher serum calcium concentrations in elderly women compared to men, although not always consistently[16,18,19], while several studies have shown that postmenopausal women have higher serum phosphate concentrations than men of similar age[5,18,20-23,33]. Recently, we showed that serum calcium and phosphate are higher in women than in men in three samples extracted from a hospital information system[24]. The findings from the current study have now confirmed sexual dimorphism both in serum calcium and phosphate concentrations at the population level.

Sex hormone actions are a potential cause of the observed sex differences in serum calcium and phosphate concentrations. We found that serum estradiol diminished the sex differences in serum calcium while serum testosterone increased sex differences in serum calcium. In sex-stratified analyses we found that serum estradiol but not testosterone was inversely associated with serum calcium in both sexes. Sex differences

in serum phosphate concentrations were not modified by serum estradiol concentrations but decreased after adjusting for serum testosterone concentrations. Sex-stratified analyses showed that serum estradiol was inversely associated with serum phosphate to a similar degree in both sexes, while the association between serum testosterone and serum phosphate appeared stronger in men than in women. Our findings are in line with a study of 59 elderly men with suppressed endogenous production of estradiol and testosterone through administration of a long-acting GnRH agonist. This study showed that depletion of estrogen but not testosterone in these men resulted in increased serum calcium concentrations and increased bone resorption markers, while both estradiol and testosterone suppression independently increased serum phosphate concentrations[34]. Thus, both estradiol and testosterone may play a role in the regulation of serum calcium and phosphate but to what extent and whether there are sex differences in the regulatory pathways remains unclear.

Calcium homeostasis is predominantly regulated at the level of the parathyroid glands, the kidneys, the intestines and bone. Both human and animal studies have found that estradiol reduces renal calcium excretion and increases intestinal calcium absorption suggesting sex differences in renal and intestinal calcium handling[35,36]. However, this is not in line with our findings of higher concentrations of serum calcium in postmenopausal women when estradiol concentrations drop, suggesting an additional regulatory mechanism. Nordin *et al*. postulated that the sexual dimorphism in calcium handling could be due to a change in PTH and increased sensitivity for the PTH action on bone after menopause[15,37]. Unfortunately, serum PTH concentrations were not available in our cohort. Interestingly, we observed that serum 25(OH)D was lower in women compared to men, while $1,25(OH)_2D_3$ was not significantly different between sexes. This may indicate that there is increased conversion of 25(OH)D into $1,25(OH)_2D_3$ in women compared to men, which could be mediated by PTH. Higher PTH concentrations in women could also explain the higher serum calcium concentrations. However, adjustments for both 25(OH) D and $1,25(OH)2D_3$ did not influence sex differences in serum calcium and phosphate.

Our data suggests that differences in sex hormones, especially serum testosterone, may partly explain the sex differences in serum phosphate above the age of 45. We cannot conclude with certainty that serum testosterone but not serum estradiol is important in the age-related sex differences of phosphate regulation since testosterone can be converted into estradiol through the activity of the enzyme aromatase cytochrome P-450[38], and therefore the action at the tissue level might be driven by estradiol as well. In sex-stratified analyses, we found that serum estradiol and testosterone are associated with serum phosphate in both sexes. This is in line with a study in community-dwelling older men, where higher serum estradiol and testosterone concentrations were independently associated with lower serum phosphate concentrations, also after adjustment for FGF23

concentrations, one of the major phosphate regulating hormones[39,40]. Meng *et al*. also found an inverse association between sex-hormones and phosphate and could not attribute this to increased bone turnover since adjustments for bone mineral density and ALP did not influence the association[39]. Estradiol is able to induce phosphaturia in a PTH-independent pathway and may be a potent stimulus for FGF23 secretion[41]. Hence, the drop in estradiol associated with menopause could lead to reduced phosphate excretion by the kidneys, which may explain the higher serum phosphate concentrations after menopause as seen in our study as well as documented by others[5,18,20-23]. Most of the studies directly assessing the influence of sex steroids on serum phosphate concentrations focused on serum estradiol despite the increasing body of evidence that suggests a relationship between serum phosphate and testosterone[40,42]. As Meng *et al*. mentioned, there have not been many studies on the influence of testosterone on phosphate handling[42]. Investigating whether testosterone has potential direct effects on phosphate handling in the kidneys and intestines and on its regulatory hormones PTH, FGF23 and klotho will lead to a greater understanding of how testosterone influences the bone-kidney axis.

Since calcium and phosphate are mainly stored in bone, we assessed sex differences in calcium and phosphate concentrations in relation to bone turnover as reflected by serum ALP. However, adjustment for ALP concentrations, which were higher in women compared to men, as is known in the postmenopausal state due to estrogen deficiency[43], did not influence our results. Although there was no availability of bone-specific ALP or other bone turnover markers, this observation suggests that a higher bone turnover in postmenopausal women compared to men does not fully explain the observed increases in serum calcium and phosphate concentrations.

Serum calcium and phosphate concentrations are associated with cardiometabolic diseases and mortality, and understanding the underlying mechanisms is a key priority. Lorenzo *et al*. have found serum calcium and the calcium-phosphate product but not phosphate to be associated with the incidence of type 2 diabetes in both sexes[44]. Moreover, Larsson *et al*. showed that a genetic predisposition to higher serum calcium concentrations was associated with increased myocardial infarction and coronary artery disease[45]. Dhingra *et al*. showed that serum phosphate concentrations, but not serum calcium concentrations, are associated with the composite endpoint of fatal and non-fatal cardiovascular disease (CVD) events in non-chronic kidney disease (CKD) subjects[22] and a recent meta-analysis by Bai *et al*. in 120,269 subjects showed serum phosphate to be associated with all-cause mortality in men but not in women[46]. Lastly, in a large prospective study, Foley *et al*. found that higher serum phosphate concentrations in young adults are associated with higher scores of coronary artery calcium concentrations[47]. The higher serum concentrations of calcium and phosphate with the resulting higher

calcium-phosphate product that we found in postmenopausal women compared to men of the same age may thus be clinically relevant. It may even be speculated that higher concentrations of serum calcium and phosphate after menopause may, in part, underlie the rise in CVD that is observed in women after menopause[48,49].

The strength of this study is the availability of data in a large number of men and women in the Rotterdam Study. The large number of covariates, including serum estradiol, testosterone, vitamin D, ALP concentrations and use of hormone replacement therapy, allowed us to study the influence of these variables on calcium and phosphate homeostasis. Despite the relatively small sex differences in serum calcium and phosphate concentrations, our findings may improve our understanding of the underlying pathways for these sex differences. Moreover, our results may be relevant in light of the increasing number of studies showing that serum concentrations of calcium and phosphate in the general populations are related to multiple diseases and mortality[5,6,12,13]. Correcting for albumin in various ways in RS-I-1 has not influenced our findings of sex-based differences in total serum calcium concentrations.

This study has several limitations. We did not have availability of serum PTH and FGF23 concentrations and urinary excretion of calcium and phosphate. We did not study ionized calcium levels, which is the biologically active fraction of calcium and the fraction regulated by PTH[50]. Moreover, we had no measurements of sex hormone binding globulin, restricting us to analyses with total serum testosterone and estradiol concentrations and not with free sex hormone concentrations. Furthermore, the fact that serum phosphate in RS-I-1 in our study was determined in a non-fasting state may have influenced some of our findings[51]. There was no data on dietary intake of calcium and phosphate which could have influenced our results as well. Moreover, data on the duration of HRT use and current HRT use was not documented, which could have obscured a possible effect of HRT on serum calcium and phosphate concentrations. Lastly, the results are not generalizable to non-Caucasian populations and despite adjustment for multiple covariates, we cannot exclude the possibility of residual confounding.

To conclude, this study demonstrates sexual dimorphism in serum calcium and phosphate concentrations with postmenopausal women having significantly higher concentrations compared to men of similar age. Serum calcium and phosphate concentrations decline with age in both sexes. Serum estradiol and testosterone are inversely associated with serum phosphate in both sexes but sex differences seem to be explained in part by serum testosterone and not by estradiol. Serum estradiol and not serum testosterone is associated with serum calcium in both sexes and may play a role in the observed sex differences in serum calcium. Vitamin D is associated with serum calcium but does not appear to explain the observed sex differences. Based on the relations between

serum calcium and phosphate concentrations and morbidity, especially cardiometabolic diseases and mortality, studies providing insight into mechanisms behind the origin of these sex differences may be of great relevance for public health and sex-based medicine.

Supplementary data and acknowledgements can be accessed through the following online resources

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10205794/

https://www.nature.com/articles/s41598-023-34800-w

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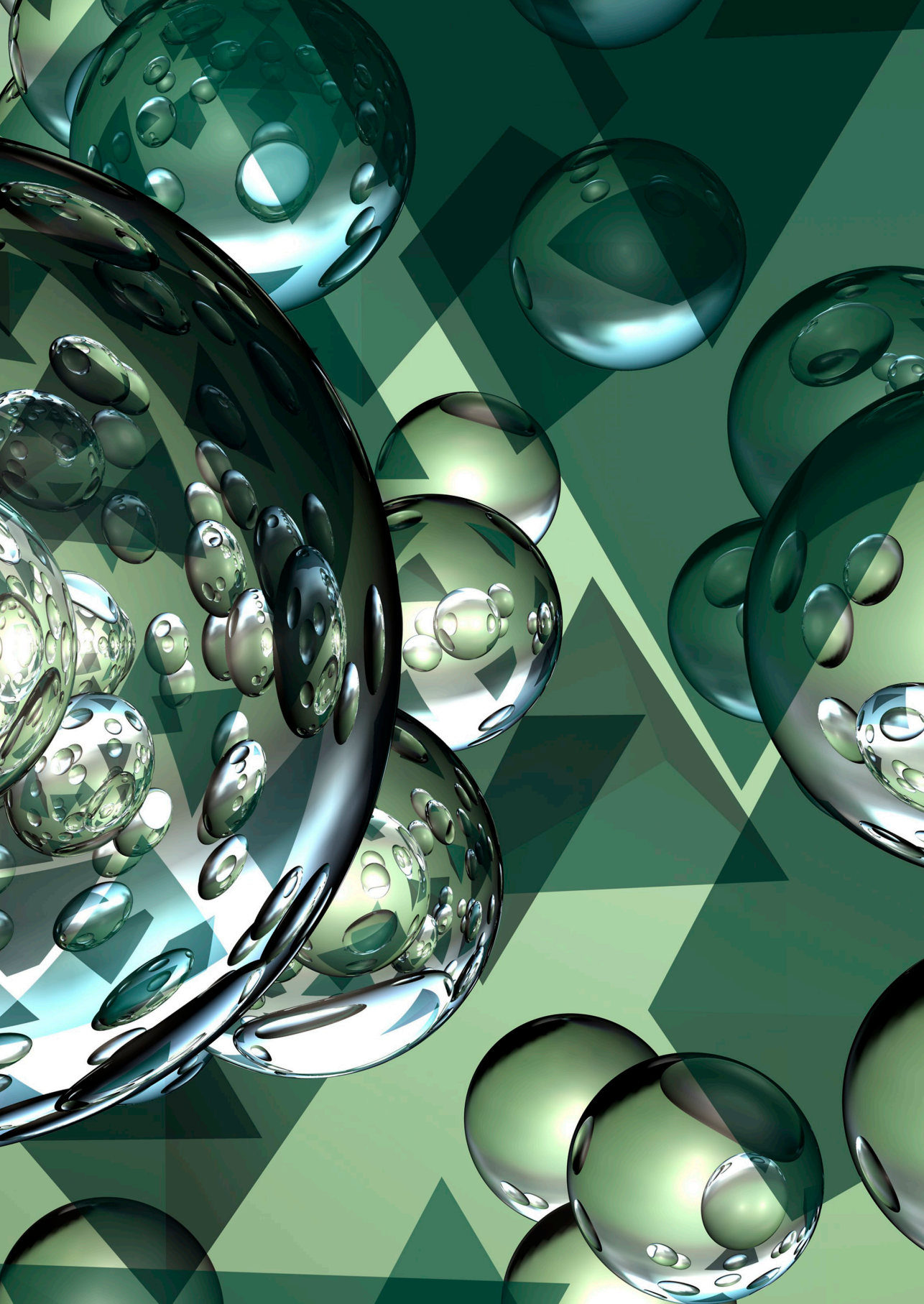
PART II

Serum phosphate in relation to BMI and diuretics use in the general population



Serum phosphate, BMI, and body composition of middle-aged and older adults: a cross-sectional association analysis and bidirectional Mendelian randomization study

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4

ABSTRACT

Background

Observational studies have reported associations between serum phosphate and body mass index (BMI) in specific clinical settings but the nature of this relation in the general population is unclear.

Objective

The aim of this study was twofold: to investigate the association between serum phosphate and BMI and body composition, and to explore evidence of causality through bidirectional one-sample Mendelian Randomization (MR) in the population-based Rotterdam Study (RS).

Methods

Observational associations between phosphate (mg/dL) and BMI, lean mass and fat percentage (fat%), estimated by DXA, were analyzed using multivariable regression models in 9,202 subjects aged 45-100 years from three RS cohorts. The role of serum leptin was examined in a subgroup of 1,089 subjects. For MR analyses, allele scores with 6 single nucleotide polymorphisms (SNPs) for phosphate and 905 SNPs for BMI were constructed in 7,983 subjects.

Results

Phosphate was inversely associated with BMI in the total population (β: -0.89; 95% CI: -1.17, -0.62), and stronger in females (β: -1.92; 95% CI: -2.20, -1.65) than in males (β: -0.37; 95% CI: -0.68, -0.06) *(P*-interaction < 0.05). Adjustment for leptin did not change results in males. In females, adjustment for leptin attenuated the association, but it was not abolished (β: -0.94; 95% CI: -1.45, -0.42). Phosphate was inversely associated with fat%, but not with lean mass, in both sexes. MR analyses suggested a causal effect of BMI on serum phosphate (β:-0.01; 95%CI:-0.02, 0.00), but not vice versa.

Conclusion

Serum phosphate was inversely associated with BMI and fat% in a population-based study of middle-aged and older adults, with a stronger effect in females than in males. Adjusting for leptin attenuated this relation in females only. MR results suggest a causal effect of BMI on phosphate but not vice versa. An underlying sex dimorphism in phosphate homeostasis should be further explored.

Keywords

Phosphate; BMI; Mendelian randomization; fat mass; lean mass; population-based cohort

INTRODUCTION

Phosphate is a widely distributed mineral ion in the body that plays an important role as an essential component of cell signaling, energy metabolism and nucleic acid synthesis[1]. Most phosphate (85%) is present within bone tissue in hydroxyapatite crystals, while 15% is found in the intracellular compartment and only 1% circulates freely in the extracellular fluids[2].

Inverse associations between serum phosphate and BMI but also between serum phosphate and Waist-to-Hip ratio (WHR), waist circumference and fat mass have been described in specific populations such as in subjects with non-morbid obesity, hypertension and metabolic syndrome[3-6]. Only a few studies have been performed at the population level. The largest study to date included 46,798 South Korean adults over 20 years of age without previous comorbidity and the authors reported a negative correlation of serum phosphate with waist circumference and BMI. After adjustment for age, sex and calcium concentrations, the association of serum phosphate with waist circumference remained robust, but the association between serum phosphate and BMI did not remain significant[7].

Several hypotheses have been proposed to explain the association between serum phosphate and BMI and body composition [8]. Phosphate concentrations are regulated predominantly by parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23)[9]. In theory, the relation between phosphate and BMI and adiposity can be explained by either serum phosphate or its regulators (PTH, FGF23 or 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or dietary phosphate) influencing adiposity, or by adiposity influencing phosphate homeostasis. Billington *et al*.[8] reported an inverse association between phosphate and fat mass in 1,676 postmenopausal females and 323 community dwelling males without active disease. This association remained significant after adjusting for age, PTH and estimated glomerular filtration rate (eGFR)[8]. Leptin, synthesized by adipocytes and strongly associated with adiposity, has been shown to function as a FGF23 secretagogue in mice and could therefore influence phosphate[10,11]. A small case-control study in 20 females undergoing bariatric surgery showed higher leptin and FGF23 concentrations in cases versus controls, but there was no difference in phosphate concentrations between the groups[10].

Recent studies have shown that phosphate is associated with all-cause mortality, cardiovascular mortality and mortality from chronic obstructive pulmonary disease (COPD) in males and progression of chronic kidney disease (CKD), among other adverse outcomes[12,13]. Moreover, conditions of low serum phosphate concentrations (hypophosphatemia) are characterized by defects at multiple levels other than bone,

such as in glucose metabolism and muscle tissue[1,5,6,14-16]. A possible phosphate-adiposity relationship may play a role in these associations and if the relationship between phosphate and mortality and morbidity can be explained by BMI, this may have consequences for health.

Due to lack of consistency and high heterogeneity of the previous findings on the association between phosphate and measures of adiposity, we aimed to investigate if serum phosphate was associated with BMI in a population-based setting with Caucasian elderly, with normal variation of both phosphate and BMI, and to investigate sex-differences, frequently reported for phosphate and several health-related outcomes[13,17]. Furthermore, we aimed to explore which body compartment drives this association and the role of potential confounders and regulators of phosphate homeostasis. For the purposes of testing causality and improving the inference of our results, Mendelian Randomization (MR) analysis was applied. MR mimics a randomized controlled trial by using natural genetic variation, which makes it less susceptible to confounding[18]. Importantly, MR analysis is considered to be unaffected by reverse causation[19]. To this end, we performed a bidirectional MR analysis using genetic variants for BMI and for phosphate as instrumental variables (IV).

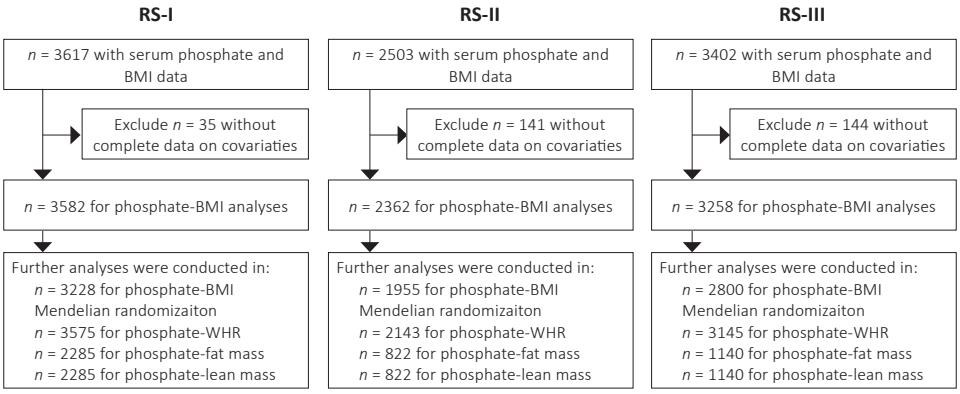
SUBJECTS AND METHODS

Study population

We performed this cross-sectional observational study and one-sample bi-directional Mendelian Randomization study in The Rotterdam Study (RS). RS is a population-based study of males and females aged 40 or more and recruited in the district of Ommoord, Rotterdam[20]. It is now composed of four cohorts named RS-I, RS-II, RS-III and RS-IV (initiated in 1989, 2000, 2005 and 2017, total n~18,000 subjects). Participants have been followed through several visits since recruitment. Rationale and design have been described previously[21]. The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands. All participants provided written informed consent to participate in the study and to obtain information from their treating physicians. For the current study, BMI, WHR and serum phosphate concentrations were assessed in the third visit of RS-I (RS-I-3, from henceforth referred to as RS-I) and in the baseline visits of RS-II and RS-III. These visits are similar in design and data collections. Measures of body composition were assessed at the fifth visit of RS-I, the third visit of RS-II and at baseline in RS-III (**Supplemental Figure 1**). A total of 3,582 participants from RS-I, 2,362 from RS-II and 3,258 from RS-III with complete information on P, BMI and covariates were included to study the observational association between serum phosphate and BMI. The total

population of 9,202 participants had a mean age of 64.9 years, range 45 – 100 years, 56.5% was female and mean BMI was 27.3 kg/m^2. Genotype data for MR analysis was available for 3,228, 1,955 and 2,800 participants from RSI, RS-II and RS-III, respectively. The total sample sizes for the analyses with WHR, fat mass and lean mass modeled as the outcome are depicted in **Figure 1**.

Figure 1 Participants flowchart summarizing sample sizes for the different analyses.



[1] MR, Mendelian randomization analyses; RS, Rotterdam Study; WHR, waist-to-hip ratio.

Clinical outcomes

Fasting blood samples were collected at the research center in which serum phosphate concentrations were determined. The amount of phosphorus determined in blood corresponds to the inorganic fraction, or phosphate, present mostly under the forms of HPO_4^{2-} and $H_2PO_4^-$ with a 4:1 ratio at a physiological pH[2]. The method for phosphate determination is based on the formation of ammonium phosphomolybdate; this compound is measured photometrically and directly proportional to phosphate concentration.

BMI (kg/m^2) was estimated from weight and height obtained in the standing position without shoes. Waist circumference was measured with a tape measure halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. WHR was calculated from these measurements. Body composition variables, namely fat mass (kg) and lean mass (kg), were determined from total body scans performed with iDXA equipment (GE Lunar)[21]. Fat percentage (Fat%) was estimated as fat mass (kg)/body weight (kg) *100. Lean mass index (LMI) was estimated as lean mass (kg)/height (cm)^2 * 100.

Confounder variables

Serum total calcium concentrations (mg/dL) were measured through a colorimetric o-cresolphthalein complexone method (Roche, Mannheim, Germany). Concentrations of serum 25-hydroxyvitamin D (25(OH)D) (nmol/L) were determined through an electrochemiluminescence immunoassay (Roche, Mannheim, Germany). Due to seasonal variability in sunlight exposure 25(OH)D concentrations were adjusted for season and year of blood sampling applying a cosinor regression method; from these models population means were obtained and individual values were adjusted[22,23]. Serum creatinine concentrations were determined through an enzymatic colorimetric assay based on the formation of sarcosine. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was applied to calculate the eGFR[24]. Serum 17β-estradiol and testosterone concentrations were determined by coat-a-count RIA (Siemens Diagnostics, Webster, TX). Due to limited amount of plasma per subject, not all hormone concentrations could be determined in all subjects.

Serum leptin concentrations were determined during the third visit of RS-I, in a random subset of participants selected as part of a separate case-cohort study (*n* = 489 males and *n* = 694 females). Leptin was quantified using multiplex immunoassay on a custom designed human multianalyte profile (Rules-Based Medicine, Austin, Texas) in a fasting blood sample. Smoking status and level of education were assessed during home interviews. Smokers were categorized as current smokers, ever smokers or never smokers. Level of education was categorized as primary, low, intermediate or high.

Genotyping

Participants were genotyped in the following platforms: Illumina HumanHap550 BeadChip, Illumina 550 duo or Illumina 610 and 660 quad SNP arrays. Variants were filtered[25,26] on call rate < 95%, minor allele frequency < 0.01 and Hardy-Weinberg Equilibrium $P < 10^{-6}$ and subsequently imputed to the Haplotype Reference consortium panel, release 1.1[27]. KING software was applied to identify highly related participants (second degree or closer), through the estimation of kinship coefficients for each pair of individuals both between and within the cohorts[28]. A kinship coefficient of 0.0884 was applied as a cut-off for second-degree relatedness.

Mendelian randomization

Mendelian randomization uses genetic instruments as instrumental variables to estimate the causal effect of a risk factor on an outcome. To this end, we constructed two genetic risk scores (GRS) to instrument BMI by adding up the BMI-related single nucleotide polymorphisms (SNPs) reported in the Genome-Wide association study (GWAS) by Yengo *et al.*[29] in 2018[30]. This meta-analysis of GWAS for BMI identified 941 GWAS significant independent SNPs from a conditional and joint multiple-SNP analysis

(COJO), including 655 SNPs from primary analysis, which explain 6,0% of the variance in BMI[29]. Genotypes for all 941 SNPs were available in our study population and we constructed two BMI GRSs: one including the 655 SNPs and one including all 941 SNPs. SNPs with an imputation quality score greater than 0.8 were included for analysis[31]. Furthermore, allele frequencies for palindromic SNPs were checked to decrease the possibility of strand coding errors. Palindromic SNPs with a minor allele frequency of more than 0.42 were discarded.

Currently, two GWAS on serum phosphate have been published: an European GWAS by Kestenbaum *et al.*[32] and a Japanese GWAS by Kanai *et al.*[33]. We constructed a GRS for serum phosphate using the 13 phosphate-related SNPs that were reported in these GWAS. In the European GWAS, the Rotterdam Study was part of the discovery sample, which could result in bias from winner's curse[34]. For this reason, GWAS summary statistics for serum phosphate were obtained from UK Biobank and the 13 SNPs were checked for GWAS significance using Neale Lab UK Biobank summary statistics[35,36]. Only the SNPs that were also GWAS significant in the UK Biobank (i.e., *P*-value $<1.0 \times 10^{-8}$) were considered for inclusion in the phosphate GRS. Imputation quality and palindromic SNPs were checked as described above.

The European GWAS on serum phosphate by Kestenbaum *et al.* (31) and the GWAS on BMI by Yengo *et al*.[29] both included the Rotterdam Study. Deriving weights from the data under analysis can result in severe bias[30]. Therefore we performed the analyses with unweighted genetic risk scores.

Statistical analysis

Differences between males and females were compared using independent T-test for continuous variables and chi-square test for categorical variables. The cross-sectional associations between phosphate concentrations (in mg/dL) with BMI and measures of body composition were examined through multivariate linear regression models with BMI, WHR, fat mass, lean mass and fat percent modeled as the dependent and serum phosphate concentrations as the independent variable. Analyses with BMI modeled as the dependent variable were performed in each of the three different cohorts separately and were meta-analyzed by applying a random effects model with Comprehensive Meta Analysis Version 3[37]. Analyses with WHR, fat mass, lean mass and fat% modeled as the dependent variable were performed in RS-III, the cohort with simultaneous measurements of laboratory data and DXA. We explored potential sex differences in the association between serum phosphate and BMI by including interaction terms of phosphate with sex in age-adjusted models, and performed sex-stratified analyses if there was evidence of a different association between serum phosphate and BMI across sexes (*P*-interaction < 0.10). The distribution of continuous variables was examined using

frequency distribution histograms and Q-Q plots.

Basic analyses were age-adjusted. All analyses were further adjusted for education level, smoking, eGFR, and for concentrations of total calcium, 25(OH)D, 17β-estradiol and testosterone. These confounders were selected based on previously reported associations with phosphate and/or with the outcomes. BMI analyses in RS-I were further adjusted for leptin concentrations, which were available in ~30% of participants from RS-I.

Since fat mass and lean mass are related to one another, we tested the correlation between fat mass and lean mass using Spearman correlation coefficients. To avoid treating highly inter-correlated variables as independent ones, analyses with fat mass and lean mass modeled as outcomes were adjusted for LMI and fat% respectively[38,39]. Since increased obesity is related to increased visceral obesity, BMI might be a confounder in the association between WHR and serum phosphate[40]. Therefore, analyses for WHR were further adjusted for BMI.

We performed several sensitivity analyses. We restricted analysis to subjects without CKD (defined as eGFR < 60 mL/(min·1.73 m^2)[24], *n* = 8125). Early stages of CKD are associated with hyperphosphaturia when there is still an adequate renal response to FGF23[41]. Furthermore, the measurements of serum phosphate and fat and lean mass were simultaneous in RS-III but not in RS-I and RS-II. We therefore proceeded to test the correlation for BMI and WHR measurements at the two different time points and we performed the body composition analysis in RS-I and RS-II as a sensitivity analysis and we meta-analyzed the results with those from RS-III. All analyses were performed with IBM SPSS software, version 21 (SPSS, Chicago, IL), Stata version 15 (STATA corp., College Station, TX) and R version 3.6.1 (Vienna, Austria).

Mendelian randomization analysis

One-sample bidirectional MR was performed in participants with data on BMI, serum phosphate, covariates and individual-level genotype data. First, we tested the three assumptions of MR[42]. To correct for multiple testing of the independence assumption, a Bonferroni correction was applied resulting in a corrected *P*-value less than 0.006 (0.05/9) (testing age, sex, education level, smoking, total calcium, 25(OH)D, eGFR, testosterone and 17β-estradiol). Next, MR analyses were performed through two stage least square regressions (2sls)[43] using Stata, with the GRS as the instrumental variable. Analyses were adjusted for age, sex and the first ten principal components (PCs) to control for population stratification. Beta, *P*-value and F-statistics were considered.

To account for potential overestimation of results due to family relatedness, 2sls MR analyses were repeated after randomly excluding first and second degree relatives,

estimated using KING software. Because previous studies have shown an inverse relationship of BMI with vitamin D deficiency, an observation that has been confirmed through MR, we performed an additional 25(OH)D adjusted MR-analysis, by including the same BMI GRS in the models as a covariate, but weighting each SNP for its effect on 25(OH)D in the study sample[44,45].

MR-Egger, the weighted median estimator and an adapted lasso regression were applied to investigate potential pleiotropy[46]. Horizontal pleiotropy would violate the exclusion-restriction condition in MR[18]. MR-Egger is able to assess directional pleiotropy[42,46] The adaptive lasso regression provides a consistent estimate while allowing less than 50% of the instruments to be invalid[47]. The weighted median approach assumes that genetic instruments representing over 50% of the weights are valid IVs[43,48]. The adjusted continuously updating estimator (CUE) was applied to account for the presence of many weak instruments[49].

RESULTS

The general characteristics of the study population, stratified by sex, are depicted in **Table 1**. More than 90% of the study population displayed serum phosphate concentrations within the normal range (2.5-4.5 mg/dL[50]). On average, females had higher concentrations of serum phosphate, total calcium and 17β-estradiol than males. Females tended to have higher BMI and higher prevalence of obesity. Leptin concentrations were higher in females than males. Males generally had higher testosterone concentrations, higher levels of education and higher values of WHR. Also, smoking was more prevalent in males than females.

Phosphate and BMI

Cross-sectional sex-combined linear regression analyses showed a significant association between serum phosphate and BMI (β-1.44; 95% CI:-1.62,-1.25; $P < 0.001$) (**Supplemental Table 1)**. Further analyses were performed sex-stratified due to evidence of an interaction between phosphate and BMI across sexes (P-interaction < 0.001). Linear regression analyses (**Table 2** and **Supplemental Table 2**) showed a significant inverse association between serum phosphate concentrations and BMI in males and a more pronounced inverse relation in females after adjustment for age, education level, smoking, total calcium, 25(OH)D, eGFR, 17β-estradiol and testosterone.

Table 1 Subject characteristics for males and females aged 45-100 years from RS-I, RS-II and RS-III of the Rotterdam Study, stratified by sex

	RS-1 (*n* = 3582)			RS-II (*n* = 2362)			RS-III (*n* = 3258)		
	Males	Females	*P*[2]	Males	Females	*P*[2]	Males	Females	*P*[2]
n	1517	2065		1063	1299		1424	1834	
Age, y	71.8 (6.7)	72.5 (7.1)	0.005	64.5 (7.5)	65.0 (8.1)	0.13	57.0 (6.6)	57.2 (7.0)	0.28
BMI, kg/m²	26.3 (3.2)	27.3 (4.4)	<0.001	26.9 (3.4)	27.4 (4.5)	0.001	27.9 (4.0)	27.6 (5.0)	0.11
Phosphate, mg/dL	3.15 (0.45)	3.62 (0.43)	<0.001	3.09 (0.44)	3.54 (0.44)	<0.001	3.22 (0.48)	3.66 (0.47)	<0.001
Normal phosphate[3], n (%)	1412 (93)	1996 (97)	<0.001	967 (91)	1266 (98)	<0.001	1330 (93)	1743 (95)	0.045
Calcium, mg/dL	9.65 (0.38)	9.80 (0.41)	<0.001	9.57 (0.35)	9.69 (0.35)	<0.001	9.81 (0.41)	9.87 (0.44)	<0.001
25(OH)D, nmol/L	61.5 (25.5)	48.0 (22.6)	<0.001	65.3 (27.8)	58.9 (27.4)	<0.001	60.5 (27.0)	60.1 (26.9)	0.67
eGFR, mL/(min·1.73 m²)	72.2 (14.6)	71.0 (13.8)	0.012	80.1 (15.1)	79.0 (15.0)	0.07	88.1 (14.6)	87.6 (14.2)	0.34
Renal impairment[4], n (%)	285 (19)	414 (20)	0.35	107 (10)	146 (11)	0.36	48 (3)	77 (4)	0.22
Current smoking, n (%)	343 (23%)	301 (15%)	<0.001	272 (26)	272 (21)	0.008	434 (31)	436 (24)	<0.001
Education, n (%)									<0.001
• **Primary education**	174 (12)	412 (20)	<0.001	69 (7)	131 (10)	<0.001	123 (9)	219 (12)	
• **Low/intermediate**	489 (32)	1024 (50)		297 (28)	772 (59)		345 (24)	810 (44)	
• **Intermediate**	575 (38)	527 (26)		415 (49)	271 (21)		481 (34)	408 (22)	
• **High/University**	279 (18)	102 (5)		282 (27)	125 (10)		475 (33)	397 (22)	
Testosterone, nmol/L	17.5 (6.1)	1.1 (0.9)	<0.001	16.3 (5.8)	0.9 (0.9)	<0.001	17.5 (5.9)	0.9 (0.5)	<0.001
17β-estradiol, pmol/L	91.4 (36.0)	33.2 (35.6)	<0.001	130 (40.6)	72.9 (63.4)	<0.001	98.3 (36.7)	121 (289)	0.001
WHR	0.98 (0.07)	0.89 (0.10)	<0.001	0.97 (0.07)	0.86 (0.08)	<0.001	0.93 (0.07)	0.83 (0.07)	<0.001
Leptin[5], ng/mL	5.53 (4.77)	17.7 (12.9)	<0.001	n/a	n/a	n/a	n/a	n/a	n/a
Fat mass[6], kg	23.3 (7.4)	28.4 (8.7)	<0.001	23.7 (7.5)	29.4 (9.4)	<0.001	24.6 (9.0)	29.7 (11.2)	<0.001
Lean mass[6], kg	55.5 (5.9)	40.4 (4.6)	<0.001	57.3 (6.0)	41.0 (4.6)	<0.001	59.5 (6.1)	41.7 (6.0)	<0.001
Fat percent[6]	27.8 (6.0)	38.8 (6.4)	<0.001	27.6 (5.9)	39.2 (6.5)	<0.001	27.3 (6.7)	39.1 (7.9)	<0.001

[1]Continuous values are displayed in mean (SD); categorical variables are displayed in absolute counts (%). 25(OH)D, 25-hydroxyvitamin D; eGFR, estimated glomerular filtration rate; RS, Rotterdam study; WHR, waist-to-hip ratio.

[2]Differences between males and females were compared using independent T-test for continuous variables and chi-square test for categorical variables.

[3]Normal phosphate was defined as a serum phosphate within the normal range (2.5-4.5 mg/dL [50]).

[4]Renal impairment was defined as eGFR < 60 mL/(min·1.73 m²)[24]

[5]Serum leptin concentrations were available in a random sample of 471 males and 618 females from RS-I.

[6]Body composition parameters were available from the fourth and second follow-up visit of RS-I and RS-II respectively and the baseline visit of RS-III.

Further adjustments of this analysis for leptin concentrations, available in ~30% of participants from RS-I (**Table 3**), did not affect results in males. In contrast, leptin adjustment attenuated but did not abolish the relation between serum phosphate concentrations and BMI in females.

Table 2 The association between serum phosphate concentrations and BMI in males and females aged 45-100 years from the Rotterdam Study[1]

	Males			Females		
	n	β (95% CI)[2]	*P*	*n*	β (95% CI)[2]	*P*
Model 1[3]	4004	-0.33 (-0.62 to-0.05)	0.022	5198	-2.22 (-2.50 to-1.95)	<0.001
Model 2[4]	4004	-0.37 (-0.68 to-0.06)	0.019	5198	-1.92 (-2.20 to-1.65)	<0.001

[1] β, beta coefficients; 95% CI, 95% confidence intervals; 25(OH)D, 25-hydroxyvitamin D; eGFR, estimated glomerular filtration rate.
[2] β and 95% CI were estimated from linear regression models and represent the change in BMI per increase in 1 mg/dL of phosphate. Analyses were performed in each of the three cohorts of the Rotterdam Study separately and estimates were meta-analyzed using a random effects meta-analysis model.
[3]Model 1: adjusted for age.
[4] Model 2: adjusted for age, smoking, education level, calcium and 25(OH)D, eGFR, testosterone and 17β-estradiol.

Table 3 The association between serum phosphate concentrations and BMI in males and females aged 61-100 years with serum leptin measurements from RS-I[1]

	Males			Females		
RS-I	*n*	β (95% CI)[2]	*P*	*n*	β (95% CI)[2]	*P*
Model 1[3]	471	-0.91 (-1.53 to-0.28)	0.005	618	-2.65 (-3.40 to-1.89)	<0.001
Model 2[4]	471	-1.14 (-1.77 to-0.51)	<0.001	618	-2.33 (-3.08 to-1.58)	<0.001
Model 3[5]	471	-1.13 (-1.67 to-0.59)	<0.001	618	-0.94 (-1.45 to-0.42)	<0.001

[1]β, beta coefficients; 95% CI, 95% confidence intervals; 25(OH)D, 25-hydroxyvitamin D ; eGFR, estimated glomerular filtration rate; RS, Rotterdam study.
[2]β and 95% CI were estimated from linear regression models and represent the change in BMI per increase in 1 mg/dL of phosphate.
[3]Model 1: adjusted for age.
[4]Model 2: adjusted for age, smoking, education level, calcium and 25(OH)D concentrations, eGFR, testosterone and 17β-estradiol.
[5]Model 3: adjusted for age, smoking, education level, calcium and 25(OH)D concentrations, eGFR, testosterone and 17β-estradiol and leptin.

Phosphate and body composition

There was a positive correlation between fat and lean mass in males and females (males: rho: 0.248; $P < 0.001$; females: rho: 0.369; $P < 0.001$). Furthermore, there was a positive correlation between BMI and WHR in males and females (males: rho: 0.616; $P < 0.001$; females: rho: 0.460; $P < 0.001$). To avoid treating highly inter-correlated variables as independent ones, analyses with fat mass modeled as the outcome were adjusted for LMI and lean mass was adjusted for fat%. Analyses with WHR modeled as the outcome were adjusted for BMI.

Table 4 displays the associations between serum phosphate and measures of body composition in RS-III, the cohort with simultaneous measurements of laboratory data and DXA. Serum phosphate concentrations were inversely associated with fat mass in females but not in males. Fat%, a measure of total adiposity, was found to be inversely associated with serum phosphate concentrations in both sexes. Serum phosphate concentrations were not significantly associated with lean mass in both sexes. WHR, a measurement of central adiposity, was not found to be associated with serum phosphate in males but females showed a significant inverse relation.

Table 4 The association between serum phosphate concentrations and measures of body composition in males and females aged 45-88 years from RS-III with measures of body composition[1]

	Males			Females		
	n	β (95% CI)[2]	*P*	*n*	β (95% CI)[2]	*P*
Fat mass						
Model 1[3]	469	-1.93 (-3.79 to-0.07)	0.042	671	-4.43 (-6.26 to-2.59)	<0.001
Model 2[4]	469	-1.29 (-3.06 to 0.48)	0.15	671	-4.11 (-5.97 to-2.24)	<0.001
Model 3[5]	469	-1.34 (-3.08 to 0.40)	0.13	671	-3.25 (-4.81 to-1.69)	<0.001
Fat percentage						
Model 1[3]	469	-1.66 (-3.02 to-0.30)	0.017	671	-2.34 (-3.63 to-1.05)	<0.001
Model 2[4]	469	-1.45 (-2.76 to-0.14)	0.031	671	-2.23 (-3.53 to-0.93)	0.001
Model 3[5]	469	-1.44 (-2.75 to-0.13)	0.032	671	-1.83 (-3.04 to-0.62)	0.003
Lean mass						
Model 1[3]	469	-0.34 (-1.56 to 0.87)	0.58	671	-1.02 (-2.00 to-0.04)	0.042
Model 2[4]	469	0.08 (-1.18 to 1.34)	0.90	671	-0.85 (-1.87 to 0.16)	0.10
Model 3[5]	469	0.07 (-1.20 to 1.33)	0.92	671	-0.28 (-1.25 to 0.69)	0.57
WHR						
Model 1[3]	1370	-0.01 (-0.014 to 0.002)	0.17	1775	-0.02 (-0.02 to-0.01)	<0.001
Model 2[4]	1370	-0.01 (-0.02 to 0.00)	0.045	1775	-0.02 (-0.03 to-0.01)	<0.001
Model 3[5]	1370	-0.004 (-0.01 to 0.003)	0.22	1775	-0.01 (-0.02 to-0.002)	0.011

[1]β, beta coefficients; 95% CI, 95% confidence intervals; 25(OH)D, 25-hydroxyvitamin D; eGFR, estimated glomerular filtration rate; LMI, lean mass index; RS, Rotterdam study; WHR, waist-to-hip ratio.
[2]β and 95% CI were estimated from linear regression models and represent the change in outcome variable per increase in 1 mg/dL of phosphate.
[3]Model 1: adjusted for age.
[4]Model 2: adjusted for age, smoking, education level, calcium and 25(OH)D concentrations, eGFR, testosterone and 17β-estradiol.
[5]Model 3: adjusted for age, smoking, education level, calcium and 25(OH)D concentrations, eGFR, testosterone and 17β-estradiol and body composition. WHR was adjusted for BMI, lean mass for fat percentage, fat mass and fat percentage was adjusted for LMI.

Sensitivity analyses

When repeating analyses in subjects without CKD, we found a borderline significant inverse relation between BMI and serum phosphate concentrations in males and a significant inverse relation in females (**Supplemental Table 3**).

BMI and WHR measurements from both visits in RS-I and RS-II proved to be positively correlated in both sexes (BMI males RS-I: rho: 0.897, RS-II: rho: 0.898; $P < 0.001$; BMI

females RS-I: rho: 0.910, RS-II: rho: 0.918; $P < 0.001$; WHR males RS-I: rho: 0.632, RS-II: rho: 0.780; $P < 0.001$; WHR females RS-I: rho: 0.462, females RS-II: rho: 0.800; $P < 0.001$). We performed the body composition analysis in RS-I and RS-II as a sensitivity analysis and meta-analyzed the results with those from RS-III (**Supplemental Table 4-7**). Serum phosphate was inversely associated with fat mass in both sexes. Serum phosphate was also significantly associated with fat% in both sexes. Serum phosphate was inversely associated with lean mass in females, but not in males. Serum phosphate was inversely associated with WHR only in females. In males, serum phosphate was inversely associated with WHR, but after adjusting for BMI and confounders, this association was no longer significant.

Genetic instruments

For the BMI GRS, 30 out of 941 SNPs were discarded due to an imputation quality score less than 0.8 and 6 SNPs were discarded as they were palindromic with an allele frequency close to 0.42. The remaining 905 SNPs from the COJO analysis and the 634 SNPs from the primary GWAS analysis were used to construct an unweighted genetic risk score (GRS) for BMI.

Concerning the first MR assumption, the 905 and 634 BMI GRSs were significantly associated with BMI, with F-statistics above 79 in all cohorts (**Supplemental Table 8**). Neither score was associated with serum phosphate. Concerning the second MR assumption, there was a significant inverse association between both the 905 and 634 BMI GRSs with 25(OH) D concentrations (**Supplemental Figure 2 and 3)**.

For the phosphate GRS, 13 phosphate-associated SNPs from a European and Japanese GWAS were considered. GWAS significance was checked in UK biobank. 6 out of 13 SNPs were independently associated with phosphate or were in high LD ($r^2 > 0.8$) with an independently associated SNP in UK Biobank. These 6 SNPs were used to construct a GRS for phosphate. The phosphate GRS was associated with serum phosphate concentrations, with F-statistics above 10 in RSI and RSII and 6.0 in RSIII **(Supplemental Table 9).** The score was not associated with BMI. The phosphate GRS was not significantly associated with potential confounders (**Supplemental Figure 4**).

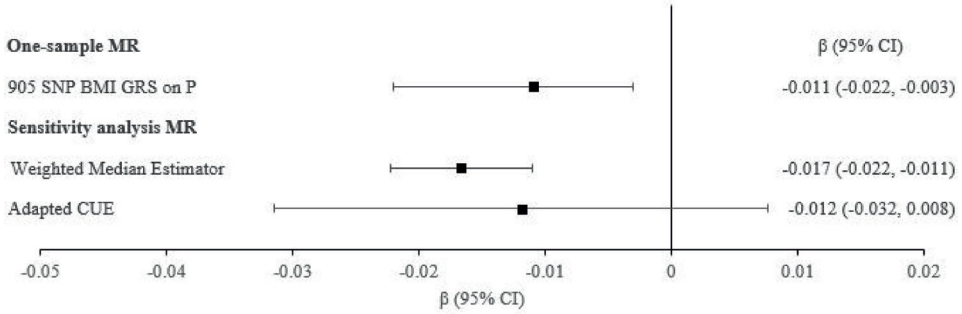
MR

The 2sls regression of BMI, instrumented by the 905 SNP GRS, on serum phosphate as outcome showed a significant causal effect of genetically determined BMI on serum phosphate (**Supplemental Table 10**). The adapted lasso regression did not show evidence for invalid instruments. MR-Egger did not show evidence for pleiotropy (intercept, $P = 0.317$). The weighted median estimator and the adapted CUE returned similar estimates (**Figure 2**). Because the BMI GRS was associated with serum 25(OH)

D, we included adjustment for genetically determined 25(OH)D by the same SNPs, to assess the likely direct (25(OH)D-independent) effects of BMI on serum phosphate. In this 25(OH)D-adjusted model, estimates were similar to the unadjusted model (data not shown). 2sls regression with the 905 SNP GRS was repeated after exclusion of first and second degree relatives and showed a significant causal effect of genetically determined BMI on serum phosphate in this group (**Supplemental Table 11**). On the other hand, 2sls regression with the 634 SNP GRS on serum phosphate showed similar estimates, but it did not reach significance (**Supplemental Table 12**).

The 2sls regression of serum phosphate, instrumented by the 6 SNP GRS, on BMI as outcome showed no evidence of a causal effect of genetically determined phosphate on BMI (**Supplemental Table 13**).

Figure 2 Mendelian randomization results for BMI and serum phosphate in males and females aged 45-100 years from the Rotterdam Study[1]



[1] β, beta coefficients; 95% CI, 95% confidence intervals; CUE: continuously updated estimator; GRS, genetic risk score; MR, Mendelian randomization; P, phosphate; SNP, single nucleotide polymorphism.

DISCUSSION

Our analyses in three cohorts of a population-based study of Caucasian elderly, consistently showed that serum phosphate concentrations were inversely associated with BMI in both sexes and this association was not influenced by education level, smoking, total calcium, 25(OH)D, eGFR and gonadal steroids. Associations were significantly stronger in females compared to males, and in a subset analysis we found leptin adjustments to attenuate but not abolish the significant results. Furthermore, phosphate was also associated with fat mass in females but not in males. Phosphate proved to be significantly associated with fat% in both sexes. With bi-directional MR analysis we found that BMI lowers phosphate (one unit higher BMI lowered phosphate by 0.01 mg/dL) but phosphate does not seem to affect BMI. Although the effect estimates from MR analyses should not be interpreted

literally, it does provide us more insight in phosphate homeostasis[19,51]. This study adds to the existing knowledge of phosphate homeostasis. Recent studies have shown that phosphate is associated with several health related outcomes[12,13]. Our findings imply that the phosphate-adiposity relationship should be taken in account when considering associations of serum phosphate and if the relation between phosphate and mortality and morbidity can be explained by BMI, this may have health consequences.

A key assumption of MR analysis is that the genetic instrument must influence the outcome only through the exposure, and not through other pathways ('horizontal pleiotropy')[18]. We performed several sensitivity analyses to test potential pleiotropic effects of the SNPs in the BMI GRS and to assess the credibility of our MR results[43]. These analyses (including adapted lasso regression, MR-Egger, weighted median approach and adjusted CUE) all returned similar estimates.

Our data suggests that BMI lowers phosphate. There are several theories that may explain this effect. A recent MR study showed that a higher BMI leads to a lower 25(OH) D level[44]. The active form of vitamin D, $1,25(OH)_2D$, is synthesized from 25(OH)D and increases phosphate absorption from the intestine. A decrease in 25(OH)D may therefore decrease phosphate absorption from the intestine. However, it must be added that, in contrast to its role on calcium homeostasis, $1,25(OH)_2D$ is likely to influence phosphate homeostasis in considerable magnitude only at the extremes of its concentration[52]. We estimated the effect of genetically predicted BMI on phosphate, controlling for the genetically determined vitamin D by the same SNPs[45]. This resulted in similar estimates with borderline significance.

A positive relation between BMI, measures of central adiposity and FGF23 concentrations has recently been described in several studies[53,54]. Hu *et al*.[55] found a positive association of serum FGF23 with abdominal obesity in 597 obese and non-obese males. In 591 postmenopausal females, both BMI and abdominal obesity were independently associated with serum intact FGF23, but there was no such association in premenopausal females (n = 411)[55]. FGF23 is the most potent phosphaturic agent discovered so far. Holecki *et al.*[56] reported a positive association between phosphate and intact and cleaved FGF23 but found no association between BMI and measures of intact and cleaved FGF23 in 3,115 elderly Polish male and female subjects[9].

We observed a more than 50% attenuation of the effect estimate in females after adjusting for leptin. Leptin derives from white adipose tissue and its levels reflect with high accuracy the amount of fat mass[11]. Consistently, leptin deficient mice display significantly higher concentrations of phosphate, calcium and 1,25-dihydroxyvitamin D than wild type mice[57]. Interestingly, leptin has been recently described as a stronger

predictor of FGF23 concentrations in females than 1,25$(OH)_2$D levels[10]. Furthermore, the existence of leptin receptors at the kidney level (proximal straight tubules, loop of Henle, distal tubules and collecting ducts) leaves room for a potential additional effect of leptin as a direct phosphaturic agent[11,58]. Thus, a phosphaturic effect of leptin through FGF23 and potentially also directly might partly explain the inverse association observed between BMI and phosphate concentrations, as reflected in the attenuation of the association after leptin adjustment in females. Previous studies have shown that leptin adjustments attenuate but not abolish the positive relation between FGF23 and body weight, BMI and fat mass in both sexes[53]. Collectively, this data support the concept that the relation between adipocytes and mineral metabolism is not fully mediated through leptin. On the other hand, leptin adjustment did not modify the association between BMI and phosphate in males, suggesting a sex dimorphism in the relation between leptin and phosphate concentrations. The potential role of 'leptin resistance', where leptin action is limited in obese states, on this sex dimorphism remains to be elucidated[59,60]. Further research will be needed to clarify our observations and to uncover the mechanisms underlying the sex dimorphism in this association.

We also considered the role of gonadal steroids as phosphate regulators as this has recently been reported. 17β-estradiol treatment has been shown to induce phosphaturia in rats, but also in females, through a PTH-independent mechanism[61-63]. Additionally, testosterone concentrations were shown to exert an important role in regulating phosphate concentrations, even with a similar magnitude as PTH[64]. We tested if 17*β*-estradiol or testosterone concentrations were playing a role as potential confounders of the observed associations. However, the adjustments for gonadal steroids did not change the association of phosphate with BMI in either sex.

Furthermore, we assessed the potential role for renal impairment, as obesity is associated with CKD progression and early stages of CKD are associated with hyperphosphaturia when there is still an adequate renal response to FGF23[41,65]. We did not find that our results were confounded by CKD, as excluding subjects with eGFR< 60 mL/(min·1.73 m^2) yielded very similar results to those obtained from the entire study population.

We observed that phosphate concentrations were related to total adiposity, as reflected by fat%. This relation was found in both sexes, but again stronger in females. The association between phosphate and WHR was mainly explained by BMI. This finding is in contrast with a previous report that suggested that phosphate was associated with fat distribution, rather than with obesity itself[3].

This study has several limitations. The population is composed of European Caucasians, precluding inference to other populations or ethnic groups. We had no availability

of serum FGF23, 1,25$(OH)_2$D nor PTH concentrations. PTH decreases phosphate by increasing renal phosphate excretion. Furthermore, it has been shown that PTH is associated with BMI in obese subjects and with fat mass in healthy postmenopausal women. Therefore, PTH could partly explain the association between phosphate and BMI[8-10,66]. Body composition was not measured in the same visit as the serum phosphate concentrations in the total research population, only in a subset, but results were mostly similar. Lastly, the F-statistic for the MR analyses with instrumented phosphate was below 10, which makes these analyses prone to weak instrument bias. Still, we found that the direction of the effect of the MR analyses with instrumented phosphate is opposite from the phenotypic analysis and from the MR analyses with instrumented BMI. Our study has several strengths, though. We were able to test and replicate findings in three large population-based cohorts, displaying normal variation of phosphate and BMI and therefore showing that this association is not restricted to subsets. Due to the sample size, sex-stratified analyses were feasible, and this highlighted the significant sex differences in our findings. Moreover, potentially important confounders could be investigated in this study. Additionally, leptin measurements were available in a subset of the population, making it possible to further explore the potential mechanisms underlying the observed associations. An important strength of our study is the availability of genotype data, which allowed us to undertake a step forward in causal inference through the implementation of MR.

In summary, we found an inverse association between serum phosphate concentrations and BMI and fat% in Caucasian elderly, with a significantly stronger effect in females compared to males. Bi-directional MR analysis indicated that BMI lowers phosphate and not the other way around. We found that serum leptin explained part of the association between phosphate and BMI in females, suggesting that fat mass is a regulator of phosphate homeostasis through production of leptin. Further research is needed to increase power and replicate our findings, especially regarding the role of leptin, and to elucidate the reasons underlying the observed sex-differences. Our findings imply that the phosphate-adiposity relationship should be taken in account when considering associations of serum phosphate.

Supplementary data and acknowledgements can be accessed through the following online resources

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8754515/

https://www.sciencedirect.com/science/article/pii/S0022316622004904?via%3Dihub#s1a

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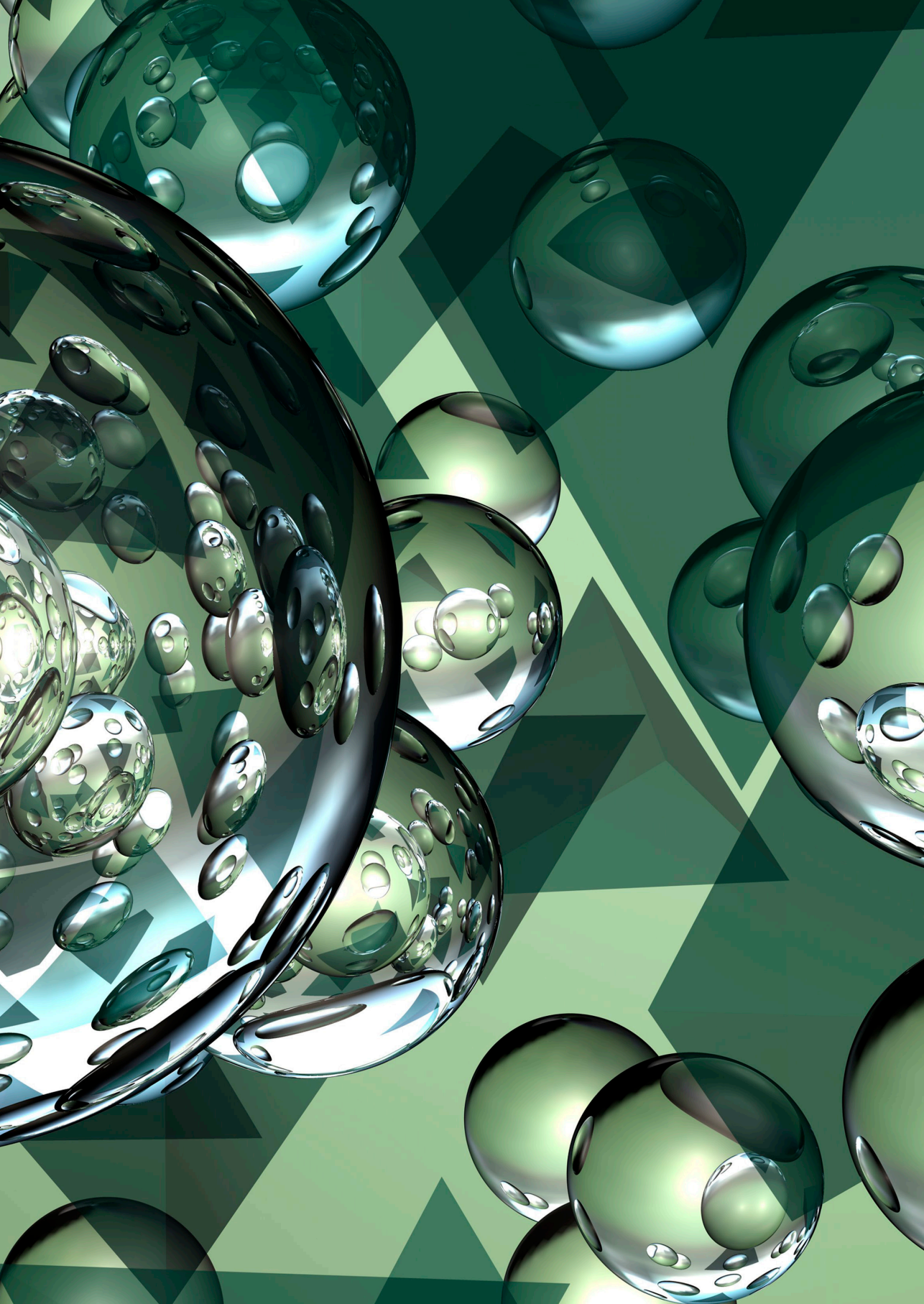

Diuretic use and serum phosphate: Rotterdam Study and UK Biobank

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ABSTRACT

Purpose

Hypophosphatemia (serum phosphate < 0.80 mmol/L) leads to musculoskeletal complaints. The most common drugs linked to hypophosphatemia are thiazide and loop diuretics but studies in the general population are lacking. Our aim was to study associations between diuretic use and serum phosphate in the Rotterdam study (RS), a population based cohort study, with replication in UK Biobank (UKBB).

Methods

Associations between thiazide and loop diuretic and serum phosphate and odds of hypophosphatemia were analyzed with cross-sectional multivariate linear and logistic regression in participants without chronic kidney disease in RS and UKBB. Analyses were adjusted for age, sex and BMI, and pooled in three RS cohorts with further adjustment for cohort and serum potassium, which was not available in UKBB.

Results

Thiazide diuretics were associated with lower serum phosphate in both sexes. This association lost significance in RS females after adjustment for BMI and in males after adjustment for serum potassium. Thiazide use increased odds of hypophosphatemia in females in both cohorts and in males in UKBB only. Loop diuretics were associated with lower serum phosphate in females but not males. Adjustment for BMI attenuated these associations. Associations between loop diuretics and increased odds of hypophosphatemia in females lost significance after BMI adjustment.

Conclusions

Thiazides, but not loop diuretics, and increased BMI and decreased serum potassium should be considered as contributing factors in subjects with hypophosphatemia. Further studies are needed to replicate the findings and elucidate the potential role of hypokalemia as a mediator of this effect.

Keywords

Phosphate, hypophosphatemia, thiazide diuretics, loop diuretics

INTRODUCTION

Hypophosphatemia, defined as a serum phosphate concentration < 0.80 mmol/L (2.5 mg/dL), is associated with various musculoskeletal conditions such as myopathy, rickets in children and osteomalacia in adults[1,2] and increased mortality in hospitalized patients[3]. Phosphate homeostasis is maintained through the control of intestinal absorption of phosphate from the diet, phosphate release from bone, and renal excretion[4]. These processes are mediated by parathyroid hormone (PTH), 1,25 dihydroxyvitamin D and fibroblast growth factor 23 (FGF23). In the kidney, phosphate is reabsorbed by the sodium-phosphate transporters 2A (NPT2A) and 2C (NPT2C) primarily at the level of the proximal tubule. Both PTH and FGF23 downregulate the expression of NPT2A and NPT2C and thereby increase renal phosphate excretion[4]. A recent review stated that thiazide and loop diuretics are among the most common drugs that have been linked to hypophosphatemia but a trial on the use of hydrochlorothiazide in hypertension did not show an effect on serum phosphate[5]. It has been hypothesized that thiazide diuretics cause renal phosphate wasting by blocking the action of carbonic anhydrase and diminishing renal tubular absorption of phosphate[6-8]. Moreover, thiazide diuretics can induce hypokalemia and hypomagnesemia, which have both been associated with renal phosphate wasting[6,9]. It is known that magnesium is necessary for PTH secretion and that hypomagnesaemia can also induce PTH resistance[10]. Loop diuretics act on the loop of Henle, that is not involved in renal phosphate handling, but they also have a mild inhibiting effect on carbonic anhydrase and can also induce hypokalemia and hypomagnesemia[6]. The effects of thiazide or loop diuretics on phosphate handling and the prevalence of hypophosphatemia have been studied mainly in specific patient groups such as hospitalized patients, patients with congestive heart failure or patients with other electrolyte disturbances[11-13]. Consequently, the reported association between thiazide or loop diuretic use and hypophosphatemia may actually reflect an association with diuretic use-related comorbidities and not a direct effect of diuretic use on serum phosphate concentration. For example, obesity is strongly associated with hypertension and cardiovascular disease, and a recent Mendelian randomization study indicated a causal association between higher BMI and lower serum phosphate[14,15]. The effect of thiazide or loop diuretics on serum phosphate in the general population and the prevalence of hypophosphatemia in users of these diuretics is currently unknown. Therefore, our aim was to study whether use of thiazide or loop diuretics is associated with serum phosphate and odds of hypophosphatemia in the Rotterdam Study (RS), a population based cohort study. UK Biobank (UKBB) was used as a replication cohort.

MATERIALS AND METHODS

All analyses were performed within RS and findings were replicated in UKBB. All participants with serum phosphate measurements and data on loop and thiazide diuretics use and BMI were included in this study. Phosphate excretion takes place primarily in the kidney, which is why patients with advanced chronic kidney disease (CKD) develop hyperphosphatemia. For this reason, we performed all analyses in participants without CKD stage 3 or higher, defined as a eGFR >60 ml/min/1.73m^2, calculated using the Chronic Kidney Disease Epidemiology Collaboration equations based on creatinine concentrations[16].

Study population

The Rotterdam Study (RS) is a population-based study of elderly males and females aged 45 years or more and recruited from the district of Ommoord, Rotterdam, the Netherlands. The first cohort initiated in 1989, named RS-I, with 7983 participants. RS-II, RS-III and RS-IV followed in 2000 (n=3011), 2005 (n=3932) and 2017 (n=4000), respectively. Participants have been followed through several visits since recruitment. All visits are similar in design and data collection. The rationale and the design of the Rotterdam Study have been described in more detail elsewhere[17].

UK biobank (UKBB) is a major biomedical database with over half a million participants who were recruited in 2006-2010 in 22 assessment centers throughout the UK. At inclusion, participants were between 40 and 69 years old. Participants consented to collection and storage of genetic, lifestyle and health information[18]. This research has been conducted using the UK Biobank Resource under application number 48264.

Assessment of serum phosphate

In the RS, fasting serum phosphate concentrations have been measured during the second follow-up visit of RS-I and the baseline visits of RS-II and RS-III using a method based on the formation of ammonium phosphomolybdate, which is directly proportional to the inorganic phosphate concentration[19].

In UKBB, serum phosphate concentrations were measured during the initial assessment visit between 2006 and 2010 and during the first repeat assessment visit between 2012 and 2013. For the current study, we analyzed data from the initial assessment visit. Serum phosphate concentrations were also measured by phosphomolybdate complex analysis (Beckman Coulter AU5800, Beckman Coulter UK, Ltd). Blood samples were drawn regardless of fasting status. There is no data on urinary phosphate excretion in RS nor in UKBB.

Medication use

In the RS, drug exposure has been monitored since January 1, 1991, through linkage with the pharmacies within the district. The following Anatomical Therapeutic Chemical codes were used: C03A, C07B, and C09BA for thiazide diuretics; C03CA for loop diuretics. In UKBB, data on medication use was collected from self-reports and during interviews. For this study, we considered all drug treatments that included either a thiazide diuretic or a loop diuretic.

Covariates

BMI (kg/m^2) was calculated from weight and height. In RS, weight and height are measured during all visits. In UKBB, weight and height were measured during the initial assessment center visit. In RS, serum sodium and potassium concentrations were determined using ion-selective electrodes. Serum total calcium concentrations (mg/dL) were determined through a colorimetric o-cresolphthalein complexone method (Roche). Serum magnesium concentrations were determined based on the complex formation of magnesium with xylidyl blue. Serum 25-hydroxyvitamin D (25(OH) D) (nmol/L) concentrations were measured using an electrochemiluminescence immunoassay (Roche). Concentrations of serum creatinine were measured through an enzymatic colorimetric assay based on the formation of sarcosine. In UKBB, serum creatinine concentrations were measured by enzymatic analysis (Beckman Coulter AU5800, Beckman Coulter UK, Ltd). Serum potassium and magnesium concentrations have not been measured in UKBB.

Statistical analysis

We explored potential sex differences in the association between diuretics use and serum phosphate by building interaction terms with sex. There was evidence of an interaction between loop diuretics use and serum phosphate across sexes (P interaction <0.001) but not between thiazide diuretics and serum phosphate (P interaction 0.277). A previous study from our group found that serum phosphate concentrations are higher in adult women compared to men[20]. Because of the interaction between loop diuretic use and serum phosphate across sexes and the differences in serum phosphate concentration between sexes across all cohorts, we performed all analyses in the total population and sex-stratified. Distribution of continuous variables was determined by visual inspection of frequency distribution histograms and Q-Q plots. Differences in continuous variables between two groups were assessed using student T tests. Differences in categorical variables between groups were tested with Chi-square test or Fisher's exact test. Multivariable linear regression models were used to study the association between serum phosphate and current thiazide and loop diuretic use. Multivariable logistic regression models were used to study the association between use of each diuretic drug with the odds of hypophosphatemia. Analyses were adjusted for age, sex and BMI.

Analyses in the RS were further adjusted for cohort. Outliers in serum phosphate of >6 standard deviations were removed from analysis. No adjustments were made for multiple testing because only two diuretics were analyzed and two large independent cohorts were used to minimize risk of chance findings.

Sensitivity analysis

To study the role of serum potassium, magnesium and 25(OH)D concentrations on the association between diuretics use and serum phosphate, we further adjusted our analyses for serum potassium and magnesium, and 25(OH)D in the RS. All analyses were performed with IBM SPSS software, version 28.0.0.0 and R version 3.6.1 (Vienna, Austria).

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalogue number NL6645 / NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. UK Biobank has been approved by the North West Multi-centre Research Ethics Committee.

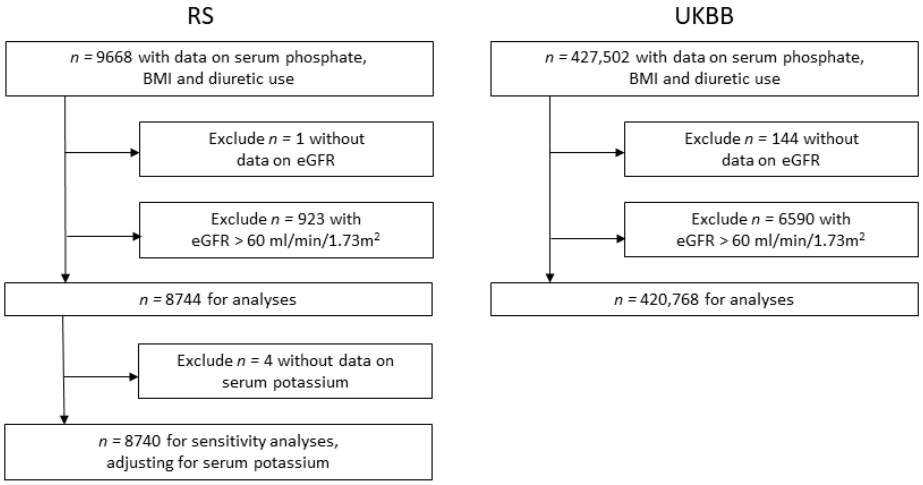
RESULTS

A total of 8,744 participants from three cohorts within RS had an eGFR >60 ml/min/1.73m^2, serum phosphate measurements and data on loop and thiazide diuretics use and BMI (**Figure 1**). The general characteristics are depicted in **Table 1**. The mean age was 63.9 years and 56.3% was female. Phosphate was higher in females than in males ($P < 0.001$) and hypophosphatemia was more prevalent in males than in females (5.7% versus 0.7%. $P < 0.001$). Thiazide and loop diuretic use was more prevalent in females with hypophosphatemia compared to females without hypophosphatemia, but not in males. BMI was higher in females with hypophosphatemia compared to females without hypophosphatemia.

In UKBB, a total of 420,768 participants had serum phosphate measurements and data on thiazide and loop diuretic use and BMI (**Figure 1**). The general characteristics of UKBB are also depicted in **Table 1**. Mean age was 56.5 years and 53.7% were female.

Similar to the RS, serum phosphate was higher in females than males (P < 0.001) and hypophosphatemia was more prevalent in males (P <0.001). BMI was higher in both males and females with hypophosphatemia compared to participants without hypophosphatemia of the same sex.

Figure 1. Participant flowchart summarizing sample sizes for the different analyses. eGFR, estimated glomerular filtration rate.



Thiazide diuretic but not loop diuretic use was more prevalent in males with hypophosphatemia compared to males without hypophosphatemia (**Table 2**). In females, thiazide diuretic use was also more prevalent in participants with hypophosphatemia compared to participants without hypophosphatemia. Loop diuretic use tended to be more prevalent in females with hypophosphatemia compared to females without hypophosphatemia, but this difference was borderline significant.

Next, we compared thiazide diuretic users with non-users for males and females separately (**Table 3**). In RS, serum phosphate concentrations were lower in thiazide diuretic users compared to non-users in both sexes. Hypophosphatemia was more prevalent in female thiazide diuretic users compared to non-users, but not in males. Moreover, in both sexes potassium and magnesium concentrations were lower in thiazide diuretic users compared to non-users, while BMI was higher. In UKBB, serum phosphate was lower and BMI higher in thiazide diuretics users compared to non-users in both sexes and prevalence of hypophosphatemia was higher in thiazide diuretic users.

Table 1. General characteristics of the study population in RS and UKBB.

	Total population	Male	Female	P-value	Normal values
RS					
N	8744	3825	4919		
Age (y)	63.9 (9.1)	63.6 (8.8)	64.1 (9.4)	0.008	
Female (%)	4919 (56.3%)				
Phosphate (mmol/L)	1.11 (0.16)	1.02 (0.15)	1.17 (0.15)	<0.001	0.80 – 1.40
Hypophosphatemia(%)	255 (2.9%)	219 (5.7%)	36 (0.7%)	<0.001	
Sodium (mmol/L)	142.1 (3.1)	142.0 (3.1)	142.2 (3.1)	<0.001	136- 145
Potassium (mmol/L)	4.34 (0.33)	4.35 (0.34)	4.35 (0.33)	0.352	3.5 – 5.1
Magnesium (mmol/L)	0.84 (0.06)	0.84 (0.06)	0.84 (0.06)	0.036	0.70 – 1.05
eGFR, ml/min/1.73m^2	82.5 (13.0)	83.3 (11.5)	82.5 (11.7)	<0.001	
BMI (kg/m2)	27.2 (4.2)	27.09 (3.6)	27.4 (4.6)	0.002	
Loop diuretics (%)	156 (1.8%)	70 (1.8%)	86 (1.7%)	0.775	
Thiazide diuretics (%)	198 (2.3%)	78 (2.0%)	120 (2.4%)	0.212	
UKBB					
N	420,768	194,103	226,665		
Age (y)	56.5 (8.1)	56.6 (8.2)	56.3 (8.0)	<0.001	
Female	226,665 (53.7%)				
Phosphate (mmol/L)	1.16 (0.16)	1.12 (0.16)	1.19 (0.15)	<0.001	
Hypophosphatemia(%)	6412 (1.5%)	5049 (2.6%)	1363 (0.6%)	<0.001	
eGFR, ml/min/1.73m^2	97.4 (14.3)	99.7 (16.3)	95.4 (12.0)	<0.001	
BMI (kg/m2)	27.4 (4.8)	27.8 (4.2)	27.0 (5.2)	<0.001	
Loop diuretic use (%)	3900 (0.9%)	1799 (0.9%)	2101 (0.9%)	0.998	
Thiazide diuretic use (%)	27,219 (6.5%)	11,560 (6.0%)	15,659 (6.9%)	<0.001	

Continuous values are displayed as mean (SD), categorical variables are displayed in absolute counts (%). Differences between males and females were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Table 2 General characteristics of males and females with and without hypophosphatemia in RS and UKBB.

	Males without HypoP	Males with HypoP	P-value	Females without HypoP	Females with HypoP	P-value
RS						
N	3606	219		4883	36	
Age, years	63.6 (8.9)	63.4 (8.0)	0.739	64.1 (9.4)	64.6 (11.5)	0.778
Phosphate, mmol/L	1.04 (0.13)	0.74 (0.05)	<0.001	1.18 (0.14)	0.76 (0.03)	<0.001
Sodium, mmol/L	142.0 (3.1)	141.6 (2.4)	0.048	142.2 (3.1)	141.4 (2.4)	0.110
Potassium, mmol/L	4.36 (0.33)	4.21 (0.39)	<0.001	4.35 (0.33)	4.19 (0.39)	0.006
Calcium, mmol/L	2.42 (0.10)	2.39 (0.11)	<0.001	2.44 (0.10)	2.45 (0.20)	0.773
Magnesium, mmol/L	0.84 (0.06)	0.84 (0.06)	0.317	0.84 (0.06)	0.93 (0.06)	0.180
Vitamine D, nmol/L	60.1 (27.8)	60.4 (28.5)	0.911	54.5 (27.2)	46.8 (27.1)	0.090
eGFR, ml/min/1.73m²	83.3 (11.5)	84.0 (11.5)	0.330	82.5 (11.7)	80.7 (12.0)	0.360
BMI (kg/m2)	27.1 (3.6)	27.4 (3.2)	0.197	27.3 (4.6)	32.9 (6.8)	<0.001
Loop diuretics (%)	68 (1.9%)	2 (0.9%)	0.436	83 (1.7%)	3 (8.3%)	0.024
Thiazide diuretics (%)	73 (2.0%)	5 (2.3%)	0.793	115 (2.4%)	5 (13.9%)	0.002
UKBB						
N	189,504	5049		225,302	1363	
Age (y)	56.7 (1.1)	55.9 (0.7)	<0.001	56.3 (8.0)	52.4 (8.2)	<0.001
Phosphate (mmol/L)	1.13 (0.15)	0.73 (0.06)	<0.001	1.20 (0.15)	0.74 (0.05)	<0.001
eGFR, ml/min/1.73m²	99.7 (16.3)	101.1 (16.2)	<0.001	95.3 (12.0)	98.3 (12.3)	<0.001
BMI (kg/m2)	27.8 (4.2)	28.6 (4.4)	<0.001	27.0 (5.2)	29.3 (6.2)	<0.001
Loop diuretic use (%)	1741 (0.9%)	58 (1.1%)	0.10	2082 (0.9%)	19 (1.4%)	0.07
Thiazide diuretic use (%)	11,182 (5.9%)	378 (7.5%)	<0.001	15,511 (6.9%)	148 (10.9%)	<0.001

Continuous values are displayed as mean (SD), categorical variables are displayed in absolute counts (%). Differences between participants with and without hypophosphatemia were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Table 3. Characteristics of thiazide diuretic users and non-users in RS and UKBB, stratified by sex.

	Male thiazide non-user	Male thiazide user	P-value	Female thiazide non-user	Female thiazide user	P-value
RS						
N	3747	78		4799	120	
Age, years	63.6 (8.9)	60.3 (6.7)	<0.001	64.1 (9.4)	62.0 (9.2)	0.014
Phosphate, mmol/L	1.02 (0.15)	0.99 (0.15)	0.034	1.17 (0.14)	1.14 (0.16)	0.010
Hypophosphatemia, n(%)	214 (5.7%)	5 (6.4%)	0.803	31 (0.6%)	5 (4.2%)	0.002
Sodium, mmol/L	142.0 (3.1)	142.1 (3.9)	0.794	142.2 (3.1)	141.7 (2.8)	0.057
Potassium, mmol/L	4.36 (0.33)	4.17 (0.34)	<0.001	4.35 (0.33)	4.14 (0.36)	<0.001
Calcium, mmol/L	2.42 (0.10)	2.43 (0.09)	0.105	2.44 (0.10)	2.47 (0.10)	0.005
Magnesium, mmol/L	0.85 (0.06)	0.82 (0.07)	0.006	0.84 (0.06)	0.82 (0.07)	0.002
Vitamine D, nmol/L	60.2 (27.8)	57.3 (27.3)	0.364	54.5 (27.2)	53.1 (26.3)	0.584
eGFR, ml/min/1.73m^2	83.2 (11.5)	86.1 (10.8)	0.028	82.5 (11.7)	82.5 (11.1)	0.995
BMI, kg/m^2	27.0 (3.6)	29.4 (4.4)	<0.001	27.3 (4.6)	30.3 (5.4)	<0.001
UKBB						
N	182,543	11,560		211,006	15,659	
Age (y)	56.3 (8.2)	61.4 (6.0)	<0.001	55.9 (8.0)	61.2 (6.0)	<0.001
Phosphate (mmol/L)	1.12 (0.16)	1.11 (0.17)	<0.001	1.20 (0.15)	1.17 (0.15)	<0.001
Hypophosphatemia(%)	4671 (2.6%)	378 (3.3%)	<0.001	1215 (0.6%)	148 (0.9%)	<0.001
eGFR, ml/min/1.73m^2	100.1 (16.3)	94.7 (16.3)	<0.001	95.6 (12.0)	91.6 (12.0)	<0.001
BMI (kg/m2)	27.7 (4.2)	29.8 (4.8)	<0.001	26.8 (5.0)	30.0 (5.7)	<0.001

Continuous values are displayed as mean (SD), categorical variables are displayed in absolute counts (%). Differences between diuretic users and non-users were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Likewise, we compared loop diuretic users with non-users in males and females separately in the RS (**Table 4**). Male loop diuretic users were older than male non-users, but phosphate concentrations and prevalence of hypophosphatemia were not significantly different. Female loop diuretic users were older, with higher BMI than female non-users. Female loop diuretic users had lower phosphate levels than female non-users and hypophosphatemia was more prevalent in the loop diuretic users. In both sexes, BMI was significantly higher in loop diuretic users compared to non-users. Serum potassium was lower in loop diuretic users in females only. Male and female loop diuretic users in UKBB were older and had higher BMI than non-users of the same sex. Serum phosphate concentrations were slightly lower in loop diuretic users compared to non-users in both sexes, but prevalence of hypophosphatemia was not significantly different.

Table 4 Characteristics of loop diuretic users and non-users in RS and UKBB, stratified by sex.

	Male loop non-user	**Male loop user**	**P-value**	**Female loop non-user**	**Female loop user**	**P-value**
RS						
N	3755	70		4833	86	
Age, years	63.4 (8.8)	71.5 (9.2)	<0.001	63.9 (9.3)	73.7 (11.2)	<0.001
Phosphate, mmol/L	1.02 (0.15)	1.04 (0.13)	0.272	1.17 (0.14)	1.10 (0.15)	<0.001
Hypophosphatemia, n(%)	217 (5.8%)	2 (2.9%)	0.436	33 (0.7%)	3 (3.5%)	0.024
Sodium, mmol/L	142.0 (3.1)	141.1 (3.2)	0.024	142.2 (3.1)	142.0 (3.1)	0.412
Potassium, mmol/L	4.35 (0.33)	4.31 (0.36)	0.326	4.35 (0.33)	4.23 (0.43)	0.014
Calcium, mmol/L	2.41 (0.10)	2.39 (0.09)	0.025	2.44 (0.10)	2.43 (0.10)	0.147
Magnesium, mmol/L	0.84 (0.06)	0.85 (0.09)	0.841	0.84 (0.06)	0.85 (0.06)	0.067
Vitamine D, nmol/L	60.3 (27.8)	54.6 (28.1)	0.095	54.7 (27.2)	38.7 (22.7)	<0.001
eGFR, ml/min/1.73m^2	83.4 (11.5)	76.6 (10.9)	<0.001	82.6 (11.5)	77.9 (17.6)	<0.001
BMI, kg/m^2	27.1 (3.6)	28.4 (3.8)	0.003	27.3 (4.6)	29.5 (5.4)	<0.001
UKBB						
N	192,304	1799		224,564	2101	
Age (y)	56.6 (8.2)	62.1 (5.8)	<0.001	56.2 (8.0)	61.4 (6.1)	<0.001
Phosphate (mmol/L)	1.12 (0.16)	1.11 (0.17)	0.004	1.19 (0.15)	1.18 (0.16)	<0.001
Hypophosphatemia(%)	4991 (2.6%)	58 (3.2%)	0.10	1344 (0.6%)	19 (0.9%)	0.07
eGFR, ml/min/1.73m^2	99.8 (16.3)	90.3 (17.3)	<0.001	95.4 (12.0)	87.7 (13.5)	<0.001
BMI (kg/m2)	27.8 (4.2)	32.5 (6.3)	<0.001	27.0 (5.1)	33.0 (7.0)	<0.001

Continuous values are displayed as mean (SD), categorical variables are displayed in absolute counts (%). Differences between diuretic users and non-users were analyzed using an independent t test for continuous variables and $\chi 2$ test for categorical variables. Abbreviations: eGFR, estimated glomerular filtration rate; BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Association between diuretic use and serum phosphate concentrations

The use of thiazide diuretics was associated with lower serum phosphate levels in the total population in both RS and UKBB. Adjustment for BMI attenuated the association in both studies but it remained significant. The inverse association between thiazide diuretic use and serum phosphate was significant in both sexes. Adjustment for BMI attenuated the association in males but it remained significant. In females, the association between thiazide diuretic use and serum phosphate lost significance in RS but not in UKBB after adjustment for BMI (RS: β: -0.019, 95%CI: -0.045 to 0.007; *P* = 0.144; UKBB: β: -0.027, 95%CI: -0.029 to -0.024; *P* <0.001) (**Table 5**).

The use of thiazide diuretics was associated with an increased odds of hypophosphatemia in the total population in both the RS and UKBB (**Table 6)**. Adjustment for BMI attenuated this association in both studies, but it remained significant in UKBB. Sex-stratified analyses showed that the use of thiazide diuretics was associated with an increased odds of hypophosphatemia in females in both RS and UKBB (RS: OR: 6.17, 95%CI: 2.27 to 16.77; *P* < 0.001; UKBB: OR: 2.47, 95%CI: 2.06 to 2.94; *P* <0.001). This association was attenuated but remained significant after adjustment for BMI. In males, this association was significant only in UKBB and it remained significant after adjustment for BMI (RS: OR: 1.15, 95%CI: 0.46 to 2.90; *P* =0.769; UKBB: OR, 1.26 ; 95%CI: 1.13 to 1.40; *P* <0.001). Sex-stratified analyses showed a significant inverse association between loop diuretic use and serum phosphate in females only (RS: β:-0.064, 95%CI:-0.095 to-0.033; *P*<0.001; UKBB: β: -0.025, 95%CI: -0.031 to -0.018; *P*<0.001). Adjustment for BMI attenuated but did not abolish this association (RS: β:-0.049, 95%CI:-0.080 to-0.019; *P*=0.002; UKBB: β: -0.007, 95%CI:-0.014 to-0.001; *P*=0.031). The association between loop diuretic use and serum phosphate was not significant in males (**Table 7**).

In females, the use of loop diuretics was associated with an increased odds of hypophosphatemia in both RS and UKBB. This association lost significance after adjustment for BMI in both studies. In males, the association between loop diuretic use and hypophosphatemia was significant in UKBB only and it lost significance after adjustment for BMI (**Table 8)**.

Table 5. Association between thiazide diuretic use and serum phosphate concentration in RS and UKBB, in the total population and stratified by sex.

	Total population			**Men**			**Women**		
	N	β (95% CI)	P-value	N	β (95% CI)	P-value	N	β (95% CI)	P-value
RS*									
Model 1	8744	-0.033 (-0.056 to-0.010)	0.005	3825	-0.042 (-0.075 to-0.009)	0.012	4919	-0.038 (-0.065 to-0.012)	0.005
Model 2	8744	-0.027 (-0.048 to-0.007)	0.009	3825	-0.038 (-0.071 to-0.005)	0.023	4919	-0.019 (-0.045 to 0.007)	0.144
UKBB									
Model 1	420,768	-0.023 (-0.025 to-0.021)	<0.001	194,103	-0.008 (-0.011 to-0.005)	<0.001	226,665	-0.035 (-0.038 to-0.033)	<0.001
Model 2	420,768	-0.016 (-0.018 to-0.014)	<0.001	194,103	-0.004 (-0.008 to-0.001)	0.005	226,665	-0.027 (-0.029 to-0.024)	<0.001

Model 1: adjusted for age and sex ; Model 2: adjusted for age, sex and BMI. *Models in RS were additionally adjusted for cohort. Abbreviations: BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Table 6. Association between thiazide diuretic use and hypophosphatemia in RS and UKBB, in the total population and stratified by sex.

	Total population			**Men**			**Women**		
	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value
RS*									
Model 1	8744	2.10 (1.08 to 4.12)	0.030	3825	1.21 (0.48 to 3.05)	0.353	4919	6.17 (2.27 to 16.77)	<0.001
Model 2	8744	1.81 (0.92 to 3.56)	0.087	3825	1.15 (0.46 to 2.90)	0.769	4919	4.00 (1.43 to 11.14)	0.008
UKBB									
Model 1	420,768	1.56 (1.42 to 1.71)	<0.001	194,103	1.38 (1.24 to 1.53)	<0.001	226,665	2.47 (2.06 to 2.94)	<0.001
Model 2	420,768	1.38 (1.25 to 1.51)	<0.001	194,103	1.26 (1.13 to 1.40)	<0.001	226,665	1.92 (1.59 to 2.29)	<0.001

Model 1: adjusted for age and sex ; Model 2: adjusted for age, sex and BMI. *Models in RS were additionally adjusted for cohort. Abbreviations: BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Table 7. Association between loop diuretic use and serum phosphate concentration in RS and UKBB, in the total population and stratified by sex.

	Total population			Men			Women		
	N	β (95% CI)	P-value	N	β (95% CI)	P-value	N	β (95% CI)	P-value
RS*									
Model 1	8744	-0.023 (-0.046 to 0.000)	0.055	3825	0.028 (-0.007 to 0.063)	0.118	4919	-0.064 (-0.095 to -0.033)	<0.001
Model 2	8744	-0.012 (-0.035 to 0.011)	0.301	3825	0.032 (-0.003 to 0.067)	0.073	4919	-0.049 (-0.080 to -0.019)	0.002
UKBB									
Model 1	420,768	-0.016 (-0.021 to -0.011)	<0.001	194,103	-0.006 (-0.014 to 0.001)	0.096	226,665	-0.025 (-0.031 to -0.018)	<0.001
Model 2	420,768	-0.003 (-0.008 to 0.002)	0.192	194,103	0.001 (-0.006 to 0.009)	0.734	226,665	-0.007 (-0.014 to -0.001)	0.031

Model 1: adjusted for age and sex ; Model 2: adjusted for age, sex and BMI. *Models in RS were additionally adjusted for cohort. Abbreviations: BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Table 8. Association between loop diuretic use and hypophosphatemia in RS and UKBB, in the total population and stratified by sex.

	Total population			Men			Women		
	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value
RS*									
Model 1	8744	1.12 (0.45 to 2.80)	0.816	3825	0.50 (0.12 to 2.07)	0.340	4919	5.09 (1.45 to 17.92)	0.011
Model 2	8744	0.97 (0.39 to 2.44)	0.950	3825	0.48 (0.12 to 1.97)	0.306	4919	3.24 (0.90 to 11.69)	0.072
UKBB									
Model 1	420,768	1.48 (1.17 to 1.85)	0.001	194,103	1.34 (1.01 to 1.72)	0.032	226,665	2.16 (1.32 to 3.31)	0.001
Model 2	420,768	1.12 (0.88 to 1.40)	0.328	194,103	1.08 (0.82 to 1.40)	0.554	226,665	1.33 (0.81 to 2.04)	0.233

Model 1: adjusted for age and sex ; Model 2: adjusted for age, sex and BMI. *Models in RS were additionally adjusted for cohort. Abbreviations: BMI, body mass index; RS, Rotterdam Study; UKBB, UK Biobank.

Sensitivity analyses

As previously mentioned, serum potassium and magnesium levels were available in the RS cohort, but not in UKBB. The association between thiazide diuretic use and serum phosphate lost significance after adjustment for serum potassium in the total RS population and in males. In RS females, the association between thiazide diuretic use and serum phosphate had already lost significance after adjustment for BMI, but the direction of the association completely reversed after further adjustment for serum potassium (**Supplementary Table 1**[21]). The association between thiazide diuretic use and odds of hypophosphatemia in the total population lost significance after adjustment for serum potassium. The association in females attenuated but remained significant after adjustment for serum potassium (OR: 3.21, 95%CI: 1.13 to 9.11; *P* = 0.029) (**Supplementary Table 2**[21]). Adjustment for serum magnesium or 25(OHD) did not change results (data not shown). Adjustment for serum potassium in the RS did not change the association between loop diuretic use and serum phosphate in females. (**Supplementary Table 3 and 4**[21]).

DISCUSSION

This study in two large population-based cohorts showed that thiazide diuretic use was associated with lower serum phosphate concentrations in both sexes and with increased odds of hypophosphatemia in females. This association may be partly explained by a difference in BMI. For loop diuretic use the results were less consistent, but there was an inverse association between loop diuretic use and serum phosphate concentrations in females. These results are in line with a randomized cross-over study in postmenopausal osteopenic women, which showed that thiazide diuretics caused a dose-dependent decrease in serum phosphate concentrations[22]. In contrast, a randomized controlled trial in healthy elderly persons did not find a significant difference in serum phosphate in thiazide diuretic users compared to placebo[23]. In linear regression analyses we show that BMI is an important confounder in the association between thiazide diuretic use and serum phosphate. In addition, serum potassium concentrations may also play a role in the observed differences in serum phosphate concentration between thiazide diuretic users and non-users. Hypophosphatemia in general can be caused by either an intracellular shift, impaired intestinal absorption of phosphate or intake, and renal phosphate wasting[22]. However, the cause of the hypophosphatemia cannot always be identified[24]. Our findings may be relevant for health care professionals who analyze and treat patients with hypophosphatemia. Thiazide diuretics have been prescribed to patients with X-linked hypophosphatemia (XLH), the most common monogenetic cause of chronic hypophosphatemia, to decrease drug-induced urinary calcium excretion and to prevent nephrocalcinosis[25]. In a small cohort study in pediatric XLH patients,

thiazide diuretic use decreased urinary calcium excretion, but it also decreased serum phosphate, which would suggest a causal relationship. Therefore, caution is warranted when prescribing thiazide diuretics to XLH patients[26].

There are several mechanisms that may explain a difference in serum phosphate between thiazide diuretic users and non-users. One pathophysiological mechanism relates to PTH. PTH regulates serum phosphate concentration by induction of bone resorption, stimulation of 1α-hydroxylase resulting in increased synthesis of calcitriol, and increasing renal phosphate excretion. The net effect is that serum phosphate levels go down[4,27]. However, in a study of postmenopausal osteopenic women thiazide diuretics decreased urinary calcium excretion without a change in PTH[22]. Unfortunately, this study did not report on the effects of diuretic use on serum phosphate concentrations. Thiazide diuretics act on the sodium chloride cotransporter NCCT in the distal convoluted tubule. Mutations in the *SLC12A3* gene encoding NCCT cause Gitelman syndrome[28]. Interestingly, in a recently published cohort study, hypophosphatemia was observed in 16% of patients with Gitelman syndrome. The authors also found an association between serum phosphate levels and TmP/GFR, indicating that the hypophosphatemia was most likely related to renal phosphate wasting. In patients with Gitelman syndrome only 6.9% had hyperparathyroidism and PTH was also not correlated with serum phosphate nor with TmP/GFR. We were not able to adjust for PTH in our analyses but these results suggest that the inverse association between thiazide diuretic use and serum phosphate concentrations is not related to increased PTH concentrations.

A second important regulator of phosphate homeostasis besides PTH is FGF23. This hormone lowers serum phosphate by increasing renal phosphate excretion and inhibiting 1α-hydroxylase[29]. Mouse studies have shown that NCCT knock out mice have higher FGF23 concentrations than wild type mice. However, fractional phosphate excretion was not different between knock out mice and wild type mice. Moreover, although the FGF23 transcript was increased in the bone of the NCCT knock out mice, thiazide diuretic treatment of osteoblasts did not result in an increase in FGF23 transcription[30]. It remains to be elucidated whether thiazide diuretic use causes an increase in FGF23 resulting in lower serum phosphate concentrations.

It has also been hypothesized that thiazide and loop diuretics affect serum phosphate concentration due to inhibition of carbonic anhydrase (CA)[7,8]. Inhibition of CA causes a decrease in secretion of H+, leading to natriuresis and diuresis[31]. CA inhibition can also cause metabolic acidosis, during which urinary phosphate excretion increases[32,33,34]. The increase in sodium delivery to the distal tubule together with a diuretic induced stimulation of the renin-angiotensin-aldosterone cascade causes an increase in distal potassium secretion, leading to hypokalemia[33]. Rat studies have shown that potassium

deficiency is associated with a decrease in sodium phosphate transporters type IIc in the proximal tubular brush border membrane[35]. In addition, a recent dietary controlled randomized trial showed that potassium supplementation leads to a decrease in FGF23 and an increase in plasma phosphate and TmP/GFR[36]. These results suggest that there may be a relation between potassium and phosphate, potentially mediated by FGF23. It has been shown that thiazide diuretics induce more hypokalemia than loop diuretics, despite the direct action of loop diuretics in Henle´s loop[37]. In the RS, thiazide diuretic users had lower serum potassium than non-users. The inverse association between thiazide diuretic use and serum phosphate in the total population and in males lost significance after adjustment for serum potassium. This association in females had already lost significance after adjustment for BMI but further adjustment for serum potassium completely reversed the direction of the association. By contrast, serum potassium was not significantly different in male loop diuretic users and in female users it was decreased but to a lesser extent. Unfortunately these findings could not be replicated in UKBB because of lack of data on serum potassium, but our results suggest that serum potassium may be a mediator in the association between thiazide diuretic use and serum phosphate.

The association between thiazide diuretic use and serum phosphate may also reflect an association between diuretic use-related comorbidities and serum phosphate. In this study we found that thiazide diuretic users from both sexes and female loop diuretic users had higher BMI than non-users of the same sex. Obesity is strongly associated with hypertension and cardiovascular disease[15]. Previous studies have reported an inverse association between BMI and serum phosphate concentrations[14,38,39]. Furthermore, a Mendelian randomization study from our group reported a suggested causal effect of BMI on serum phosphate[14]. A higher BMI may lead to higher FGF23 concentrations, possibly through leptin, resulting in increased renal phosphate excretion[14,40-43]. Also, it has been shown that BMI is associated with PTH[44,45]. Therefore, PTH may play a role in the association between BMI and phosphate. Indeed, adjustment for BMI attenuated the association between thiazide diuretic use and serum phosphate concentrations in males in RS and UKBB and in females in UKBB. In RS, the association between thiazide diuretic use and serum phosphate lost significance in females after adjustment for BMI. The association between serum phosphate and odds of hypophosphatemia attenuated but remained significant in both sexes in UKBB and in females in RS after adjustment for BMI. These results suggest that BMI is an important confounder in these associations. Lastly, the association between loop diuretic use and serum phosphate was only significant in females. A possible explanation for this sex-difference is that female loop diuretic users had lower serum 25(OH)D concentrations than female non-users, although results did not change after adjustment for 25(OH)D. It is likely that 25(OH)D only influences phosphate homeostasis at the extremes of its concentrations[46].

This study has several limitation. We performed cross-sectional analyses, which refrains us from drawing conclusions on the effect of initiation, duration and dose of diuretics use on serum phosphate concentrations. Due to the design of the study no adjustments were made for multiple testing, however a Bonferroni correction would not have changed the significance for thiazide diuretics but it would for loop diuretics, supporting our conclusion that thiazide diuretics and not loop diuretics are associated with serum phosphate in men. As mentioned previously, blood samples were drawn non-fasting in UKBB. Moreover, medication use in UKBB is self-reported, which is susceptible to errors. Interestingly, despite lower age in UKBB, loop diuretic use was lower and thiazide diuretic use was higher in UKBB compared to RS, suggesting national differences in prescription. Still, results from association analyses were similar. We had no availability of PTH or FGF23 concentrations and also no data on comorbidities or use of other drugs. Also, serum potassium has not been measured in UKBB. However, drugs that are known to affect serum phosphate (e.g., antriretrovirals, anticancer drugs and calcineurin inhibitors) are not often used in the general population and we are not aware of any disorders that are associated with both diuretic use and serum phosphate. Malnutrition or alcohol abuse as the cause of lower phosphate concentrations in diuretic users seems unlikely because in our study BMI was higher and there is no literature data showing that use of diuretics is associated with alcohol abuse. There are several strengths, such as the availability of two large well-characterized population-based cohorts and the ability to replicate the findings.

In conclusion, this study in two population-based cohorts showed that thiazide diuretic users have lower serum phosphate concentrations than non-users in both sexes. Hypophosphatemia was more prevalent in female thiazide diuretics users than in female non-users. Loop diuretic use was not associated with serum phosphate nor with hypophosphatemia in males, while in females there was an inverse association between loop diuretic use and serum phosphate. BMI appears to be an important confounder of these associations, while serum potassium may be a mediator. Thiazide diuretic use, but not loop diuretics, and increased BMI and decreased serum potassium should be considered as a contributing factor in patients with hypophosphatemia. Further studies are needed to replicate the findings and elucidate the potential role of hypokalemia as a mediator of this effect.

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Data availability

RS data can be obtained upon request. Requests should be directed towards the management team of the Rotterdam Study (datamanagement.ergo@erasmusmc.nl), which has a protocol for approving data requests. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository. The data from UK Biobank is open source and available to researchers after acceptance of a research proposal and payment of an access fee.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Association between thiazide diuretic use and serum phosphate concentration in RS, in the total population and stratified by sex, additionally adjusted for serum potassium.

	Total population			Men			Women		
	N	β (95% CI)	P-value	N	β (95% CI)	P-value	N	β (95% CI)	P-value
RS									
Model 1	8744	-0.033 (-0.056 to -0.010)	0.005	3825	-0.042 (-0.075 to -0.009)	0.012	4919	-0.038 (-0.065 to -0.012)	0.005
Model 2	8744	-0.027 (-0.048 to -0.007)	0.009	3825	-0.038 (-0.071 to -0.005)	0.023	4919	-0.019 (-0.045 to 0.007)	0.144
Model 3	8740	-0.010 (-0.031 to 0.010)	0.308	3823	-0.023 (-0.056 to 0.009)	0.157	4917	-0.001 (-0.027 to 0.024)	0.913

Model 1: adjusted for age, sex and cohort ; Model 2: adjusted for age, sex, cohort and BMI. Model 3: adjusted for age, cohort, BMI and serum potassium. Abbreviations: BMI, body mass index; RS, Rotterdam Study.

Supplementary Table 2. Association between thiazide diuretic use and odds of hypophosphatemia in RS, in the total population and stratified by sex, additionally adjusted for serum potassium.

	Total population			Men			Women		
	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value
RS									
Model 1	8744	2.10 (1.08 to 4.12)	0.030	3825	1.21 (0.48 to 3.05)	0.353	4919	6.17 (2.27 to 16.77)	<0.001
Model 2	8744	1.81 (0.92 to 3.56)	0.087	3825	1.15 (0.46 to 2.90)	0.769	4919	4.00 (1.43 to 11.14)	0.008
Model 3	8740	1.40 (0.71 to 2.78)	0.332	3823	0.89 (0.35 to 2.26)	0.886	4917	3.21 (1.13 to 9.11)	0.029

Model 1: adjusted for age, sex and cohort ; Model 2: adjusted for age, sex, cohort and BMI. Model 3: adjusted for age, cohort, BMI and serum potassium. Abbreviations: BMI, body mass index; RS, Rotterdam Study

Supplementary Table 3. Association between loop diuretic use and serum phosphate concentration in RS, in the total population and stratified by sex, additionally adjusted for serum potassium.

	Total population			**Men**			**Women**		
	N	β (95% CI)	P-value	N	β (95% CI)	P-value	N	β (95% CI)	P-value
RS									
Model 1	8744	-0.023 (-0.046 to 0.000)	0.055	3825	0.028 (-0.007 to 0.063)	0.118	4919	-0.064 (-0.095 to -0.033)	<0.001
Model 2	8744	-0.012 (-0.035 to 0.011)	0.301	3825	0.032 (-0.003 to 0.067)	0.073	4919	-0.049 (-0.080 to -0.019)	0.002
Model 3	8740	-0.005 (-0.028 to 0.018)	0.664	3823	0.036 (0.002 to 0.070)	0.040	4917	-0.039 (-0.069 to -0.009)	0.010

Model 1: adjusted for age, sex and cohort ; Model 2: adjusted for age, sex, cohort and BMI. Model 3: adjusted for age, cohort, BMI and serum potassium. Abbreviations: BMI, body mass index; RS, Rotterdam Study.

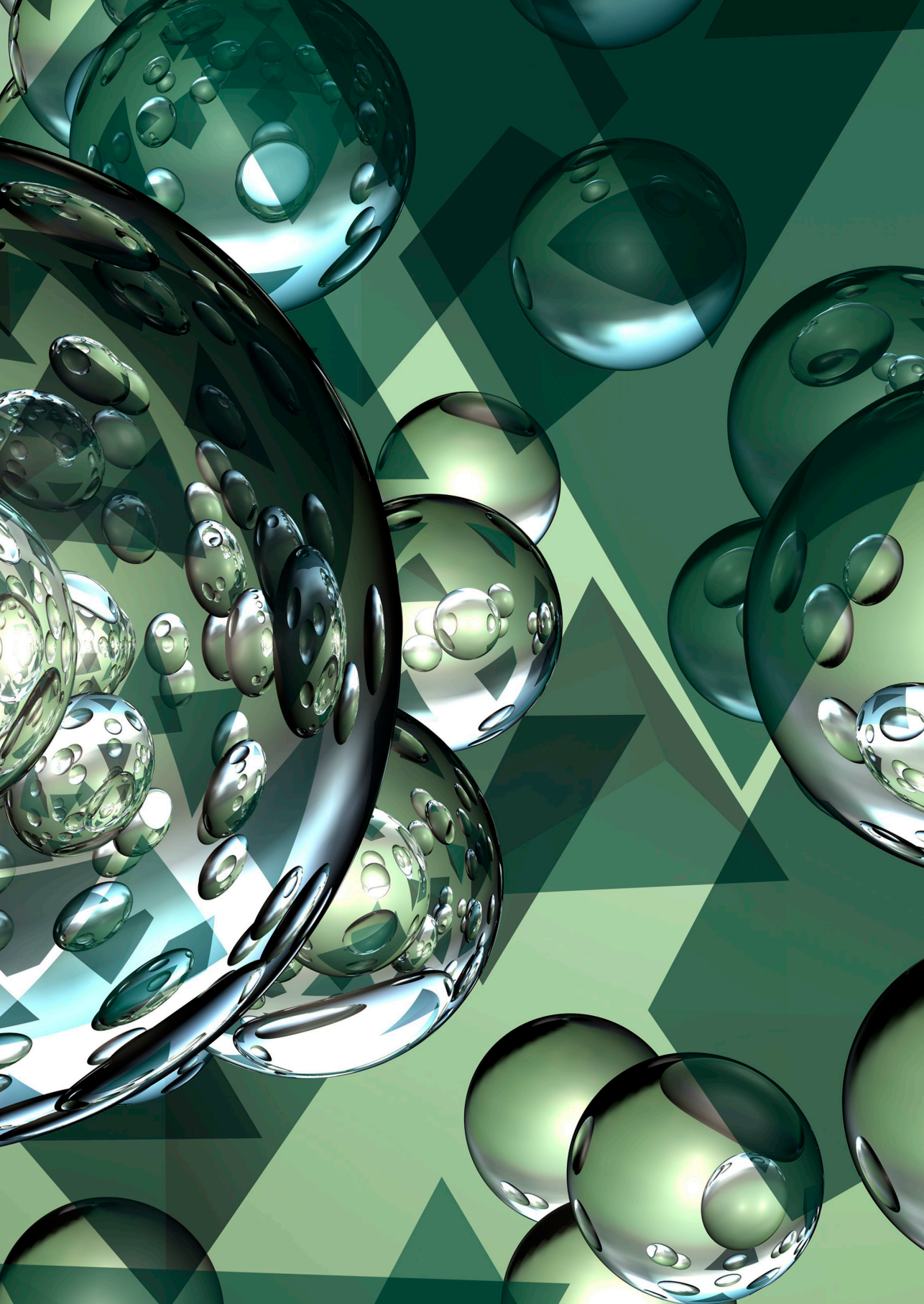
Supplementary Table 4. Association between loop diuretic use and odds of hypophosphatemia in RS, in the total population and stratified by sex, additionally adjusted for serum potassium.

	Total population			**Men**			**Women**		
	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value	N	OR (95% CI)	P-value
RS									
Model 1	8744	1.12 (0.45 to 2.80)	0.816	3825	0.50 (0.12 to 2.07)	0.340	4919	5.09 (1.45 to 17.92)	0.011
Model 2	8744	0.97 (0.39 to 2.44)	0.950	3825	0.48 (0.12 to 1.97)	0.306	4919	3.24 (0.90 to 11.69)	0.072
Model 3	8740	0.88 (0.35 to 2.23)	0.791	3823	0.45 (0.11 to 1.85)	0.266	4917	2.62 (0.72 to 9.57)	0.145

Model 1: adjusted for age, sex and cohort ; Model 2: adjusted for age, sex, cohort and BMI. Model 3: adjusted for age, cohort, BMI and serum potassium. Abbreviations: BMI, body mass index; RS, Rotterdam Study.

PART III

Causes of hypophosphatemia



Tumor-induced osteomalacia: a systematic clinical review of 895 cases

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ABSTRACT

Tumor-induced osteomalacia (TIO) is a rare and largely underdiagnosed paraneoplastic condition. Previous reviews often reported incomplete data on clinical aspects, diagnosis or prognosis. The aim of this study was to present a systematic clinical review of all published cases of TIO. A search was conducted in Pubmed, Embase, Web of Science from inception until April 23rd, 2020. We selected case reports and case series of patients diagnosed with TIO, with information on tumor localization and serum phosphate concentration. Two reviewers independently extracted data on biochemical and clinical characteristics including bone involvement, tumor localization and treatment. 468 articles with 895 unique TIO cases were included. Median age was 46 years (range 2 – 90 years) and 58.3% were males. Hypophosphatemia and inappropriately low or normal 1,25-dihydroxyvitamin D levels, characteristic for TIO, were present in 98% of cases. Median tumor size was 2.7 cm (range 0.5 to 25.0 cm). Serum fibroblast growth factor 23 was related to tumor size ($r=0.344$, $P < 0.001$). In 32% of the cases the tumor was detected by physical examination. Data on bone phenotype confirmed skeletal involvement: 62% of cases with BMD data had a T-score of the lumbar spine ≤-2.5 (n=61/99) and a fracture was reported in at least 39% of all cases (n=346/895). Diagnostic delay was longer than 2 years in more than 80% of cases. 10% were reported to be malignant at histology. In conclusion, TIO is a debilitating disease characterized by a long diagnostic delay leading to metabolic disturbances and skeletal impairment. Increasing awareness of TIO should decrease its diagnostic delay and the clinical consequences.

Keywords

Tumor-induced osteomalacia, FGF23, Osteomalacia, Rickets, Hypophosphatemia, Fracture

INTRODUCTION

The rare and debilitating condition of tumor-induced osteomalacia (TIO), also known as oncogenic or oncogenous osteomalacia, is nowadays more frequently recognized, especially since its pathophysiological mechanisms are better understood. In this paraneoplastic disease, the tumor secretes phosphaturic factors known as "phosphatonins"[1-3], amongst which fibroblast growth factor 23 (FGF23) is the most frequently found, leading to the cardinal features of the disease: hypophosphatemia from renal phosphate wasting, reduced 1,25-dihydroxyvitamin D concentration through inhibition of its synthesis, rickets in children and osteomalacia in adults, with diffuse bone pain, fractures and muscle weakness. Radical resection of the responsible tumor leads to a rapid normalization of biochemical parameters and to marked improvement or resolution of the symptoms[4]. The majority of the tumors are benign and arise from mesenchymal tissue[5].

Since the initial presentation can be misleading or non-specific, TIO still remains a diagnostic and therapeutic challenge despite the progress made in its understanding. Moreover, the lack of serum phosphate measurement in many standard comprehensive chemistry panels contributes to its delayed diagnosis[6]. In addition, the causative tumor can develop anywhere in the body and can be small enough to elude even our modern imaging techniques[7,8]. The consequence can be a long diagnostic delay, leading sometimes to a dramatic outcome with multiple fractures and severe disability. In order to identify tumor mass, a stepwise imaging approach using functional and anatomic imaging is suggested. Several techniques are being used to detect the tumor, including but not limited to computed tomography (CT) or magnetic resonance (MR), 18F-fluorodeoxyglucose (FDG) PET/CT, Technetium 99m octreotide, scintigraphy/SPECT/CT and Gallium-68 (^{68}Ga)-DOTATATE PET/CT. Recent studies have demonstrated that 68Ga-DOTATATE PET/CT shows the greatest accuracy in TIO localization[6,9,10].

A few clinical reviews aimed to investigate the clinical profile of TIO but they presented significant limitations: the authors included cases of acquired hypophosphatemic rickets / osteomalacia even if the tumor was not found[4,7,11-14]. The overall aim of this review is to carefully describe the clinical and biochemical aspects and the bone phenotype of TIO by conducting a complete analysis of all published cases between 1947 and 2020. Although in clinical practice the diagnosis often can be suspected based on the clinical characteristics and the biochemical findings, we chose a more precise approach. To avoid uncertain or incorrect diagnoses, the current study focuses only on cases where the causative tumor was localized and treatment led to cure or marked improvement of the patient's condition. While performing our review, another systematic review was published on patients with a clinical diagnosis of TIO[15]. Our approach enabled us to

describe more precisely the clinical presentation and the localization of the responsible tumor in patients with TIO.

METHODS

Data Sources and Searches

This review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement (PRISMA)[16]. Due to the nature of our research question and inclusion criteria, we did not perform a separate risk of bias assessment. We searched Pubmed, Embase and Web of Science from inception to April 23rd 2020, without language restrictions. We screened references lists of included articles. Search terms focused on tumor-induced osteomalacia and hypophosphatemic rickets (**Appendix Table 1)**.

Study selection

Two reviewers screened all articles and abstracts of conferences containing descriptions of clinical cases of TIO. The criteria appraised to select cases were as follows: acquired hypophosphatemia due to renal phosphate wasting with a reported serum phosphate level before treatment, no known family history of osteomalacia, reported localization of the causative tumor, and cure after appropriate treatment or at least clear-cut improvement (in terms of clinical and biochemical parameters or in the amount of medical treatment needed). Case reports on linear sebaceous naevi[17,18], von Recklinghausen disease[19,20], fibrous dysplasia of bone [21], McCune-Albright syndrome [22], and hematological malignancies[23-25] were excluded. These conditions are known to be potentially associated with hypophosphatemia but are better described as "tumor-induced osteomalacia like syndrome"[14]. In addition, some cases were published more than once (80 reports) and only the first publication was included.

Data Extraction

Two investigators reviewed all cases. When available, the following information was collected: demographic data (age, sex); data about the disease (time from the first symptoms to diagnosis and treatment, outcome after treatment, duration of follow-up); data about the tumor (location, signs at physical examination pointing out to the tumor, presence or absence of symptoms that could be attributed to the tumor directly, techniques used for localization, histology, malignant features and size of the tumor); biochemical data (serum phosphate, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D concentration, plasma FGF23 concentration and tubular maximal phosphate resorption/glomerular filtration rate (TmP/GFR)) and data about bone health (occurrence of fractures and bone mineral density (BMD) at the lumbar spine, total hip and femoral

neck assessed by dual-energy X-ray absorptiometry). TmP/GFR, if not directly available in the manuscript, was calculated using the nomogram of Walton & Bijvoet[26]. FGF23 values were recorded, taking into account the assay used and the normal range. Since FGF23 was measured with several different commercial or in-house kits and since they are not interconvertible[27], we expressed FGF23 values as the times of the upper limit of the respective normal ranges. Serum phosphate and TmP/GFR were expressed in mmol/L and 1,25-dihydroxyvitamin D concentrations were expressed in pmol/L with the conversion factors of 0.323 and 2.4 to convert from mg/dL and pg/mL, respectively. 25-hydroxyvitamin D concentrations were expressed in ng/mL. A tumor was considered malignant when either mentioned as such or when an invasive behavior was evident, such as the occurrence of metastases.

Statistical analysis

Data are presented as mean ± SD or median with the interquartile range according to distribution. Statistical tests used for comparisons were the Mann Whitney-U test, Kruskal-Wallis test and chi-square test for homogeneity. Spearman correlation analysis was used to analyze the correlations among continuous variables. All analyses were performed with IBM SPSS software, version 25 (SPSS, Chicago, IL),

RESULTS

We identified 468 articles on 895 unique cases of TIO, spanning a period from 1947 until 2020 (**Figure 1**), and they are reported in Online Resource 1. Less than 5% of cases were published before 1980 and more than 70% were published in the last decade, suggesting that the disease becomes more frequently recognized with time (**Appendix Figure 1**).

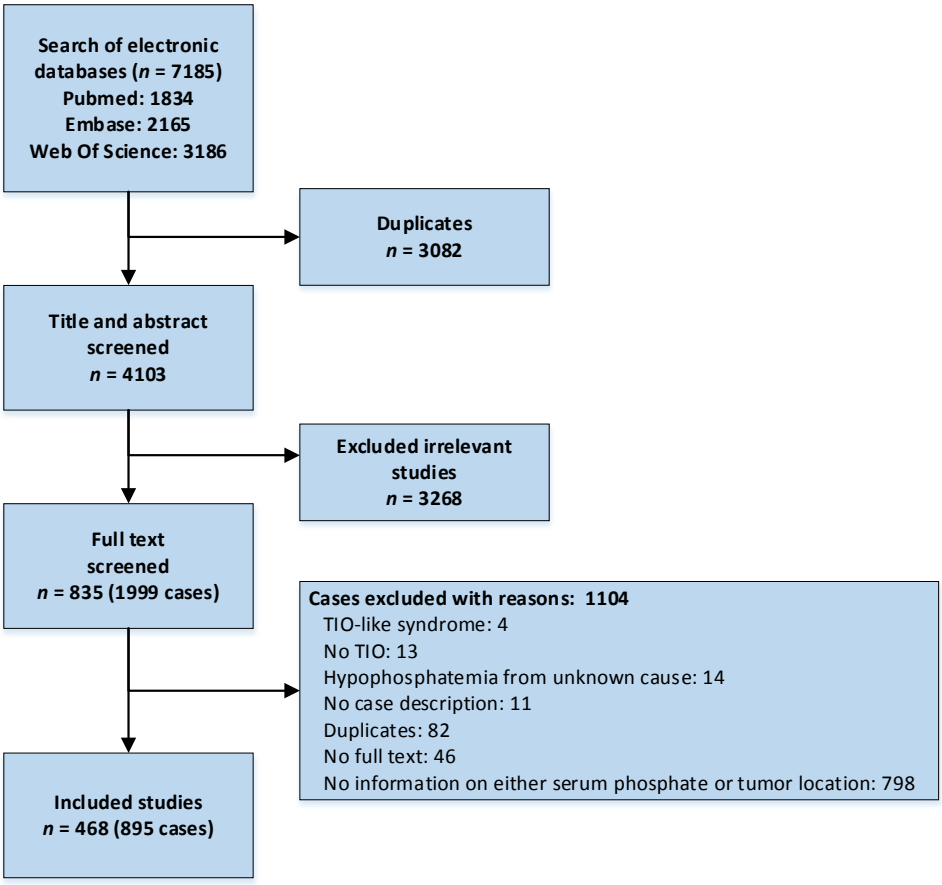
Demographic data

The disease occurred at any age from 9 months to 90 years with a median age at the time of diagnosis of 46 years. The majority of patients were aged between 30 and 60 years with a peak between 45 and 55 years of age (**Appendix Figure 2)**. Of the 858 cases with information on sex, 41.7% were females and 58.3% were males.

Biochemical data

In adults, mean serum phosphate was 0.48 ± 0.15 mmol/L (N=829; normal range: 0.74–1.52) [28] and 96.1% had hypophosphatemia. The median TmP/GFR was 0.36 mmol/L (from 0.02 to 1.80; N=357; normal range: 0.81–1.36) [26]. In patients under 18 years old, these values were 0.59 ± 0.26 mmol/L (N=38) and 0.37 mmol/L (from 0.14 to 0.92; N=14), respectively.

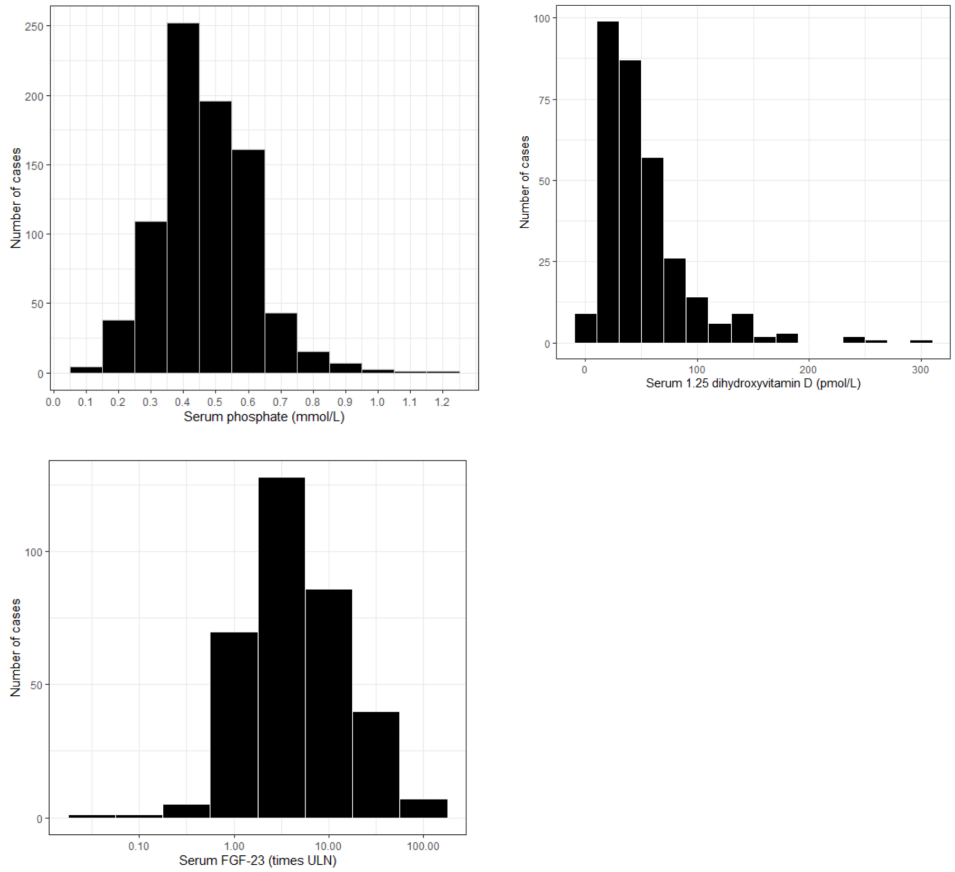
Figure 1. Flow diagram of the search



One of the cardinal features in TIO is a low or inappropriately normal circulating concentration of 1,25-dihydroxyvitamin D since hypophosphatemia is expected to stimulate renal 1α-hydroxylase which will increase 1,25-dihydroxyvitamin D production. The median value of 1,25-dihydroxyvitamin D concentration was 51.1 pmol/L (from undetectable to 301.6 pmol/L; N=337; normal range: 50–155) (**Figure 2)**. More than 60% of the patients had 1,25-dihydroxyvitamin D values below the lower limit of the normal range. About one third of the values lied within the normal range and 8 patients had elevated values of 1,25-dihydroxyvitamin D, of whom 5 patients had elevated values of parathyroid hormone with low or normal calcium levels. Thus, more than 97% of 1,25-dihydroxyvitamin D values were low or inappropriately normal. There was a significant positive correlation between 1,25-dihydroxyvitamin D and serum phosphate level (r=0.227; *P*<0.001; N=337). The median value of 25-hydroxyvitamin D concentration

was 23.5 ng/mL (from undetectable to 150.0 ng/mL; N=373; normal range: 25-80). The median FGF23 value was 3.75 times the upper limit of the normal range (xULN) (0.0-162; N=346). Over 80% of the results lied between more than 1 to 21 times xULN (**Figure 2**).

Figure. 2 Distribution of serum phosphate, 1.25 (OH)2 D levels and the times of the upper limit of normal FGF-23 among adults with TIO.



Histograms showing the distribution of serum phosphate in mmol/L (A), serum 1.25(OH)2 Vitamine D in pmol/L (B) and the times of the upper limit of normal of FGF-23 (C). Normal range for serum phosphate: 0.74 – 1.52 mmol/L. Normal range for serum 1.25(OH)2 Vitamin D: 50-155 pmol/L. Abbreviations: FGF-23, Fibroblast growth factor 23; ULN, upper limit of normal.

In 31 of 346 cases, FGF23 was below the upper limit of normal, varying from 0.02 to 0.99 times the upper limit of normal. Four of these cases had a tumor size smaller than 1.5 cm, eight cases had a tumor size 1.5-3.0 cm. We found a positive correlation between FGF23 (xULN) and tumor size (r=0.344, *P*<0.001; N=130) (**Appendix Figure 3**). There was a significant negative correlation between FGF23 and serum phosphate (r=-0.114 *P*=0.034;

N=346) and between FGF23 and Tmp/GFR (r=-0.243; *P*=0.001; N=187). The correlation between FGF23 and serum 1,25-dihydroxyvitamin D was not significant (*P*=0.443). FGF-23 levels were not always reported. Therefore, we analyzed the differences between cases who had FGF-23 measured with cases for which no FGF23 levels were reported. Interestingly, tumor size was significantly smaller in patients who had FGF23 measurements (median tumor size 2.5 cm) than in patients without reported FGF23 measurements (median tumor size 2.9 cm; *P*=0.013). Moreover, TmP/GFR was slightly lower in patients without FGF23 measurements (*P*=0.010). The diagnostic delay seemed slightly shorter in the cases with FGF23 measurements, but this was not significant **(Appendix Table 2)**.

Tumor characteristics

We considered five regions: head and neck, trunk, pelvis, upper and lower limbs. The two most frequent localizations were the lower limbs (46.4%) and the head and neck area (25.7%) (**Figure 3)**. The median size of the tumor was 2.7 cm ranging from 0.5 to 25 cm (N=416). No relationship was found between tumor size and serum phosphate levels (*P*=0.12), TmP/GFR (*P*=0.63) or 1,25-dihydroxyvitamin D levels (*P*=0.44). However, a weak but positive correlation was found between the size of the tumor and diagnostic delay (r=0.113, *P* =0.033; N=354). **Table 1** depicts the differences between tumor sizes ranging from tumors smaller than 1.5 cm to larger than 5.0 cm. The diagnostic delay and FGF23 levels were significantly different between the different tumor sizes.

Since these tumors can be found anywhere in the body, we determined how frequently they could be detected readily on just clinical examination. From the total of 895 descriptions, 494 cases reported a physical examination. 160 tumors, i.e. 32.4% of the cases with reported physical examination and 17.9% of the total number of cases, were reported to have been identified at physical examination. For tumors larger than 5.0 cm, physical examination identified the tumor in 59.5% of the cases with a reported physical examination (N=25/42) and 42.4% of the total number of cases (N=25/59). In the 461 adult cases with physical examination, external tumors were slightly larger than tumors that were not identified at physical examination, with a median size of 3.0 cm (range: 0.5-15.0 cm) vs. 2.5 cm (0.6–15.0 cm), respectively (*P*<0.001) (**Table 2**). The time to diagnosis was not significantly different between external tumors and tumors that were not identified by physical examination: 4.0 years (0.2–25 years) vs. 3.0 years (0.1–42 years), respectively (*P*=0.06). **Appendix Figure 4** depicts the identification of external and internal tumors by year of publication.

In this review, 56 tumors out of 579 (9.7%) were reported to be malignant at histology. The size of the tumor tended to be larger in the group with a malignant tumor: 5.3 cm (1.2–15.0 cm) vs. 2.5 cm (0.5–15 cm) (*P*<0.001) **(Appendix Table 3).** Smaller tumors (i.e. less than 3 cm) were almost always benign (97.9%). Malignant tumors were most

frequently diagnosed as PMT (phosphaturic mesenchymal tumor) (19.6%), followed by osteosarcomas (7.1%) while amongst PMTs, 2.4% were found to be malignant.

Tumors responsible for TIO are very heterogeneous. However, since the work of Weidner et al.[5] and Folpe et al.[29] who also reviewed previous cases, it appears that the majority was considered to be a PMT (65.4%; N=527/806), followed by hemangiopericytoma (9.1%; N=81/806), giant cell tumor (2.9%; N=26/806) and hemangioma (2.1%; N=19/806). If one considers only the cases published after the publication by Folpe et al. in 2004, PMT account for 75.5% (N=512/678) of the cases. The second most frequent histological diagnoses was hemangiopericytoma (6.3%; N=48/678), followed by giant cell tumor (2.6%; N=20/678)

Figure. 3 Localization of the tumor

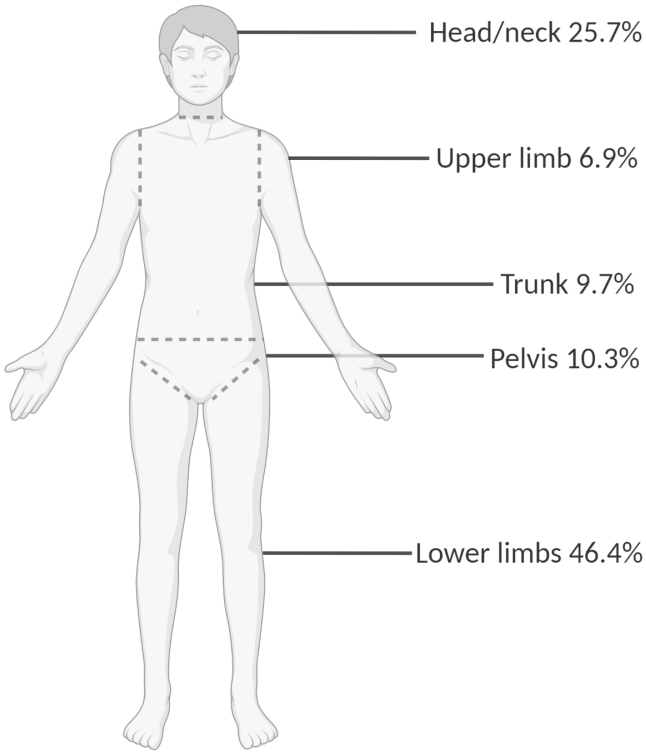


Table 1 Differences between tumors of different sizes in adults

	Tumor <1.5cm		Tumor 1.5-3cm		Tumor 3-5cm		Tumor>5cm		
	N		N		N		N		P-value*
Age, years	63	46.0 (21.0, 73.0)	151	46.0 (18.0, 76.0	125	46.0 (19.0, 73.0)	54	46.0 (18.0, 90.0)	0.999
Diagnostic delay, years	53	3.0 (0.25, 19.0)	128	3.0 (0.5, 20.0)	109	3.0 (0.17, 27.0)	48	5.5 (0.17, 21.0)	0.032
Phosphate, mmol/L	63	0.48 (0.23, 0.87)	151	0.48 (0.19, 0.90)	125	0.45 (0.11, 1.03)	54	0.42 (0.16, 0.90)	0.320
TmP/GFR, mmol/L	31	0.36 (0.09, 1.30)	72	0.39 (0.02, 1.25)	52	0.36 (0.09, 1.50)	17	0.31 (0.11, 0.74)	0.768
Calcium, mmol/L	49	2.25 (1.10, 2.86)	106	2.28 (2.00, 2.64))	88	2.26 (1.98, 10.50)	37	2.30 (1.95, 2.70)	0.146
FGF23 xULN	24	2.53 (0.56, 15.56)	57	2.78 (0.13, 48.67)	35	5.06 (0.28, 139.19)	12	4.66 (1.03, 45.00)	0.005
BMD T-score L1-L4	13	-2.7 (-4.5,-1.3)	36	-3.1 (-5.9, 0.1)	12	-2.2 (-4.8,-1.1)	10	-2.8 (-6.9,-1.2))	0.861
BMD T-score Total hip	3	-2.6 (-3.9,-2.5)	14	-2.5 (-5.0,-0.8)	1	-	3	-2.9 (-5.9,-1.2)	0.936
BMD T-score femoral neck	6	-3.4 (-5.3,-0.8)	19	-2.8 (-4.5, 0.4)	6	-2.8 (-4.6,-2.2)	5	-3.0 (-3.6,-1.5)	0.749
Tumor localization	63		151		125		54		0.073
- **Lower limb**		41 (61.5%)		78 (51.7%)		55 (44.0%)		25 (46.3%)	
- **Upper limb**		2 (3.2%)		7 (4.6%)		12 (9.6%)		6 (11.1%)	
- **Head/neck**		10 (15.9%)		39 (25.8%)		30 (24.0%)		9 (16.7%)	
- **Trunk**		6 (9.5%)		10 (6.6%)		12 (9.6%)		10 (5.6%)	
- **Pelvis**		4 (6.3%)		17 (11.3%)		15 (12.0%)		3 (5.6%)	
- **Multiple locations**		0 (0.0%)		0 (0.0%)		1 (0.8%)		1 (1.9%)	

* Differences between groups were tested using Mann Whitney-U test, Kruskal-Wallis test and chi-square test for homogeneity. Continous data is presented as median (range). Categorical data is presented as count (%). Abbreviations: BMD, bone mineral density; FGF23, Fibroblast growth factor 23; TmP/GFR, maximum tubular reabsorption rate of phosphate; xULN, times the upper limit of normal.

Table 2 Differences between external and internal* tumors in adults

	External		Internal*		
	N		N		P value†
Age, years	154	44.5 (18.0, 79.0)	307	47.0 (18.0, 90.0)	0.057
Diagnostic delay, years	141	4.0 (0.2, 25.0)	235	3.0 (0.1, 42.0)	0.055
Tumor size, cm	105	3.0 (0.5, 15.0)	150	2.5 (0.6, 15.0)	<0.001
Phosphate, mmol/L	154	0.47 (0.11, 1.20)	307	0.45 (0.10, 0.90)	0.991
Calcium, mmol/L	115	2.30 (1.95, 2.77)	211	2.25 (1.27, 2.90)	0.070
TmP/GFR, mmol/L	62	0.26 (0.03, 0.74)	130	0.36 (0.02, 1.80)	0.004
FGF23 ULN	30	3.8 (0.3, 63.0)	115	3.7 (0.3, 62.6)	0.938
BMD T-score L1-L4	19	-3.5 (-5.5,-0.5)	38	-2.7 (-5.9, 3.7)	0.106
BMD T-score total hip	6	-2.7 (-5.9,-0.9)	14	-3.3 (-5.0,-0.2)	0.444
BMD T-score femoral neck	11	-3.6 (-7.4,-1.7)	23	-2.5 (-5.3, 1.6)	0.019
Tumor localization	154		307		
- **Lower limb**		108 (35.2%)		72 (46.8%)	<0.001
- **Upper limb**		13 (4.2%)		16 (10.4%)	
- **Head/neck**		88 (28.7%)		44 (28.6%)	
- **Trunk**		49 (16.0%)		13 (8.4%)	
- **Pelvis**		45 (14.7%)		8 (5.2%)	
- **Multiple locations**		4 (1.3%)		1 (0.6%)	

*Internal tumors were defined as tumors that were not identified at physical examination. † Differences between groups were tested using Mann Whitney-U test, Kruskal-Wallis test and chi-square test for homogeneity. Continuous data is presented as median (range). Categorical data is presented as count (%). Abbreviations: BMD, bone mineral density; FGF23, Fibroblast growth factor 23; TmP/GFR, maximum tubular reabsorption rate of phosphate; ULN, upper limit of normal.

Clinical characteristics

Apart from the symptoms secondary to osteomalacia, such as proximal muscle weakness or pain, a tumor can lead by its anatomical location to specific symptoms such as tenderness, paresthesias, paresis anosmia, nasal obstruction or epistaxis. Out of 895 cases, 74 experienced local symptoms indicating that a thorough medical history and physical examination can give a clue as to the tumor localization in at least 8.3% of the cases. Interestingly, in 42 out of 216 cases (19.4%) with a tumor in the head and neck region, local symptoms were described that might in theory have led to tumor localization on clinical grounds. In 32.4% of cases where a physical examination was performed, a tumor was identified (N=160/494). Out of the 334 cases were a tumor could not be identified on physical examination, 9.3% experienced local symptoms such as redness, swelling, pain. Taken together, 21.3% (n=191/895) of the tumors in the total population or 38.7% (N=191/494) of the cases with a reported physical examination could have been localized just on clinical grounds.

Symptoms secondary to osteomalacia, such as proximal muscle weakness or pain, occurred in at least 89.9% of cases and seemed more prevalent in cases with tumors

located in the upper and lower limb and head and neck region (91.9%, 91.3% and 90.9%, respectively) than in the trunk and pelvis (82.8% and 85.9%, respectively). However, the occurrence of symptoms secondary to osteomalacia was not significantly different between localizations of the tumor (*P*=0.108).

We next evaluated how many cases described the occurrence of fractures. 422 out of 895 cases evaluated and described whether a fracture occurred. Strikingly, 346 of these patients (82.0% of 422 and 38.7% of the total) had one or more recent fractures (exact frequency not always described). 41.0% (N=142/346) suffered one fracture, 24.5% (N=85/346) suffered two fractures, 25.4% (N=88/346) suffered three fractures and 9.0% (N=31/346) suffered four or more fractures. The majority of these patients had a hip fracture (56.6%, N=196), followed by a rib fracture (51.4%, N=178) and a vertebral fracture (38.4%, N=133).

TIO is by definition a defect in bone mineralization. In addition, we were interested in the effect of the disease on bone density. The lumbar spine BMD was described in only 95 adult cases (11.4%). Strikingly, 57 out of these 99 cases (60.0%) had a T-score below -2.5. The BMD of the femoral neck was analyzed only in 47 cases (5.7%), but also here 32 cases (68.1%) had a T-score below-2.5. No correlation was found between lumbar spine BMD and serum phosphate (N=95), FGF23 (N=47) or tumor size (N=71).

We next evaluated which imaging techniques were used to detect the tumors. Most tumors were located using MRI (36.8%), followed by CT scan (29.3%), ^{18}F-fluoro-deoxy-glucose (FDG)-PET/CT (15.8%) and X-ray (10.3%). ^{68}Gallium-DOTATATE,-DOTANOC and –DOTATOC PET/CT scanning together located 20.3% of the tumors. Other less commonly used techniques were nuclear imaging, ultrasound and angiography.

The time gap from the initial patient's complaints to the diagnosis and cure was highly variable, ranging from 0.1 to 42 years with a median delay of 3.5 years. Only 20% of case were correctly diagnosed within 2 years while in 30% it took between 5 and 25 years to diagnose TIO (**Appendix Figure 5)**. In addition, physicians did not perform better during the last decade despite the availability of modern imaging armamentarium. Before 2010, the median delay was 3.5 years, after 2010, the median delay was still 3.4 years (*P*=0.608).

We found a positive correlation between tumor size and the diagnostic delay (r = 0.113, *P*=0.033, N=354). In addition, **Table 2** shows that both diagnostic delay and FGF23 levels increase with tumor size. Specifically, tumors >5cm had a median diagnostic delay of 5.5 years, while tumors 1.5-3.0 cm had a median diagnostic delay of 3.0 years (*P*=0.015).

Treatment

The administration of phosphate supplementation to treat the hypophosphatemia was reported in 461/895 cases. 81.6% (N=376/461) of these cases were treated with phosphate supplementation. Calcitriol or alphacalcidol was administered in 76.9% (N=332/432) of cases with information on calcitriol use. Apart from phosphate and active vitamin D, most tumors were treated with surgery only (84.2%, N=754/829) or with a combination of surgery and chemotherapy or radiotherapy (4.2%). Chemotherapy, radiotherapy and radiofrequency ablation as a single treatment entity were used in a few cases (N=27). In all cases, there was an improvement or a normalization of the biochemical parameters. Malignant tumors were managed with surgery only (50.0%), chemotherapy and/or radiotherapy in addition to surgery (32.0%), chemotherapy only (14.0%) or radiotherapy only (2.0%).

A follow-up duration of more than 6 months was reported in 325 cases. The duration of follow-up in this group varied from 0.6 to 26 years with a median of 2.0 years. Recurrence was reported in 16.1% (N=50/310) among which 20.0% were malignant and 80.0% were benign. As expected, the proportion of local recurrence was significantly higher in malignant tumors (36.4% vs. 15.8%; *P*=0.034; N=224). Out of the 754 surgically treated cases, 279 had a minimal follow-up duration of 6 months. 14.2% of these patients (N=38/267) reported recurrence of disease during follow-up.

DISCUSSION

In this review, we describe the clinical and biochemical aspects and the bone phenotype of published TIO cases. Several reviews about TIO have been published previously [4,7,11,13,14,30,31], but they provide incomplete clinical details because they did not include a systematic review of all the published cases of TIO until now. Recently, Rendina et al. performed a systematic review on TIO, but they also included cases with only a clinical diagnosis of TIO[32]. In the current study, we included cases with a reported serum phosphate before treatment, a localized tumor and cure after appropriate treatment or at least marked improvement, thereby excluding cases with uncertain or incorrect diagnoses. Careful inspection of each published case – including also non-English publications – , allowed us to better characterize the clinical features of this condition and to draw conclusions on tumor size, diagnostic delay and bone phenotype hitherto not reported.

Our data show that the majority of TIO occurs in adults, mainly in their forties and fifties. However, also cases have been described in very young children implicating that if in childhood inherited conditions cannot be demonstrated, TIO should be suspected and

looked for. We found that TIO was more prevalent in males with 58% of cases, which is in agreement with the recent systematic review performed by Rendina et al, but in contrast with previous literature[4,15,33].

The diagnosis of TIO depends on clinical evaluation, biochemical testing and tumor identification[34]. According to our review, a thorough clinical examination can result in identification of the tumor. Patients were often aware of a lump or a growth for many years and in some cases the tumors caused symptoms other than those related to osteomalacia, mostly due to the compression of adjacent vascular or neurological structures. In sum, in nearly 40% of the patients with reports on clinical evaluation, the tumor could have been localized just on clinical grounds. This is quite a high percentage and has not been reported before, but the literature on this topic is scarce. Shah et al. reported on 163 previously published cases of TIO in the head and neck region in whom the tumor was localized using clinical evaluation in 16.7% of cases (N=22/131)[35]. Similarly, we found that 19.4% of cases with a tumor in the head and neck region reported local symptoms. It should be noted that we do not have information on the timing of the tumor detection during clinical examination. Our review only includes cases with a localized tumor and it is possible that identification of the tumor on imaging aided in linking local symptoms to the tumor location.

It has been well known that bone involvement represents one of the most important metabolic consequences of TIO. Transiliac bone biopsy samples from TIO subjects showed a severe condition of osteomalacia with low mineralized bone volume, low mineralized trabecular thickness, and a significant increase in the heterogeneity of mineralization[36,37]. We found that 60.0% of cases with a report on BMD showed a low bone mass (based on T-scores ≤- 2.5). The tremendously high rate of fractures in our study population (82% reported one or more fractures) confirms how bone fragility represents a hallmark of the disease. This is in line with previous reports on the severe decrease of BMD at any investigated skeletal sites[38,39] and impairment of bone microarchitecture and strength[40] with consequent fragility fractures which frequently occur in weight-bearing bones[38,41]. The fracture rate reported in our study is higher than the fracture rate reported by Rendina et al. (56% in men and 48% in women), which may be explained by the fact that we applied different inclusion criteria or because we distinguish between articles that reported on fracture occurrence and articles that did not. Nevertheless, our findings imply that the bone phenotype is a hallmark of the disease and confirm the need for bone evaluation in patients with chronic hypophosphatemia[42].

As to the location of the tumors, they were found anywhere in the body but most often in the lower limbs (46.4%) and in the head and neck area (25.7%). This is in line with the publication by Rendina et al., who reported that 56% of the tumors in men and 49%

of the tumors in women were located in the lower extremities and 27% of the tumors in men and 34% of the tumors in women were located in the head and neck region[4,7]. These two sites should thus be explored with scrutiny if the tumor is not readily found. The size of the tumors varied considerably, from 0.5 cm to 25 cm, but the largest ones (>5 cm) were found predominantly in the lower limbs. Our analyses showed that tumor size did not correlate with serum phosphate, TmP/GFR or 1,25-dihydroxyvitamin D but it was significantly correlated with FGF23 levels. This is in line with previous literature and might suggest that in an untreated patient, an increase in FGF23 level over time could represent an increase in tumor size[43]. This finding underlines the importance of timely tumor localization and removal.

The diagnostic delay for TIO has been reported to range from 2.5 to 28 years[34]. Similarly, we found a median time gap between the initial presentation and the tumor related treatment of 3.5 years with a range from 0.1 to 25 years. Surprisingly, this delay has not fallen significantly in the last decade despite the availability of very powerful imaging techniques, suggesting that the awareness of the condition is still a major determinant in the celerity of the diagnosis. This is supported by the finding that the diagnostic delay was not significantly different for external tumors compared to internal tumors. Interestingly, tumors larger than 5 cm had a median diagnostic delay of 5.5 years, while tumors less than 5 cm had a median diagnostic delay of 3.0 years. In addition, tumors were larger in the group without FGF23 measurements. Possible explanations for the longer delay in the group with the largest tumor are: the lack of FGF23 measurements, and the lack of experience of the treating physician in recognizing this condition.

Hypophosphatemia and renal phosphate wasting are cardinal features in TIO. However, since serum phosphate is not always included in the standard chemistry panel, failure to identify or recognize low serum phosphate levels can delay the diagnosis. For this reason, we make the case that serum phosphate should always be measured in case of muscle weakness or pain, bone pain and fatigue, with or without fractures. Measurement of serum FGF23 is relevant in any patient with hypophosphatemia and renal phosphate wasting[34], and in the current review it was never found below the limit of the normal range. However, in a few cases FGF23 was within the normal range, which is still abnormal in the setting of hypophosphatemia[44]. A possible explanation for this is the role of other inhibitors of phosphate transport such as Fibroblast growth factor 7 or secreted frizzled-related protein 4[45,46]. Measurement of intact FGF23 seems to be the most specific and sensitive test in TIO[47]. However, raised FGF23 cannot definitively establish the diagnosis of TIO since there is a considerable overlap in FGF-23 levels between TIO and inherited conditions of hypophosphataemia[44], TIO-like syndromes[48], recent renal transplantation[49], and drug-related hypophosphatemia, mainly due to intravenous iron[50-52] Still, FGF23 levels are generally higher in TIO than in XLH and other

causes of hypophosphatemia[44]. Rendina et al. found that serum levels of intact FGF23 were higher in patients with a localized tumor compared to patients in whom the tumor was not identified, which raises the question whether these patients were correctly diagnosed as TIO.

Histology has been simplified since the work of Weidner et al.[5] in 1987 and Folpe et al.[29] in 2004. Prior to these studies, many tumors were qualified as e.g., hemangiopericytomas, hemangiomas, giant cell tumors and osteoblastomas. Both Weidner et al. and Folpe et al. reviewed the histology of tumors that were involved in TIO and concluded that many of them could be reclassified as PMT, a morphologically distinct entity that can be further classified into four groups: mixed connective tissue, osteoblastoma-like, non-ossifying fibroma-like and ossifying fibroma-like. Nevertheless, not all causative tumors fall in this group, as revised diagnoses still included other tumors e.g., hemangiopericytomas and hemangiomas[5,29]. In this review, we found that the majority of causative tumors was classified as PMT (65.4%), followed by hemangiopericytoma and giant cell tumor. When we considered only the cases that were published after the publication by Folpe et al. in 2004[29], PMT account for 75.5% of the cases. These results demonstrate that the classification of the tumors has improved and that the histopathologic characteristics of PMT are recognized. Nevertheless, the histological classification of PMT does not entirely correlate with the clinical presentation, since not all PMTs lead to hypophosphatemia[53]. In this review, most of the tumors were benign. It's important to underline that about 10% of cases were reported to be malignant at histology. This percentage is higher than reported by Rendina et al., but not all TIO cases included in our review reported information on histology. Amongst PMTs, we found that only 2.4% was malignant. Also non-PMT malignant tumors can secrete FGF23 leading to hypophosphatemia with renal phosphate wasting[54,55]. This may worsen the burden of symptoms associated with the cancer and may warrant additional treatment with phosphate supplements and active vitamin D metabolites.

The only curative treatment for TIO is removal of the tumor. Hence, localization of the tumor is very important. Often a multimodality approach is necessary, including functional imaging followed by anatomical imaging of suspicious areas[34,56]. Due to the nature of this study we cannot draw conclusions on the best performing imaging techniques in TIO. It is likely that negative results of imaging modalities were not reported, which precludes us from making statements about the sensitivity and specificity of the different imaging techniques that are available. Our data suggest that in the last two decades more internal tumors were found, implying that localization techniques have become more efficient. However, despite the availability of advanced imaging techniques, we did not see a decrease in diagnostic delay in the last ten years, indicating that recognition of the clinical characteristics of TIO needs to be improved.

As to the treatment, complete surgical resection with wide margins when feasible is the optimal treatment[57]. Incomplete resection is usually not sufficient for full resolution of the symptoms and can lead to recurrence. In those cases, phosphate and active metabolite of vitamin D supplements can be continued[14]. We found that the majority of patients were treated with surgery only (84.2%) or with a combination of surgery and chemotherapy or radiotherapy (4.2%). However, it should be noted that we only included cases in whom the tumor was localized and tumor related treatment led to marked improvement or cure. Cases in whom a tumor was identified but tumor related treatment was not initiated, not possible or did not result in improvement in clinical and biochemical parameters, were not included. Therefore, we cannot draw any conclusions on treatment effectiveness. Furthermore, we found that 81.6% of cases were treated with phosphate supplementation and 76.9% of cases were treated with calcitriol before any tumor related treatment, which shows that the importance of medical therapy in TIO before tumor removal is well recognized.

Lastly, we found a recurrence rate of 14.2% in surgically treated patients. This is higher than what was recently reported by Li. et al.[57], who observed a recurrence rate of 7.8% after primary surgery in 230 retrospectively analysed patients from a single tertiary hospital in China. Nevertheless, although many cases did not report on follow-up duration or recurrence, these results suggest that surgical treatment for TIO is highly effective and should be considered in all patients with localized tumors.

Our review has a few weaknesses. We have only included cases with a reported localized tumor and we excluded cases where the tumor could not be located despite exhaustive investigation[58-60]. It is likely that these cases do not get published, which may have resulted in an overrepresentation of the less challenging cases in our review. Second, the variables assessed in this study are extracted from the case reports. For this reason, there is a considerable amount of missing data. Moreover, there is a variability related to the different assays of FGF23 used in terms of sensitivity and/or specificity, and unknown timing of the measurements and/or influence of medical therapy, which could have concealed or decreased correlations for example between FGF23 and phosphate and tumor size. Third, the concept of disease duration may be elusive as symptoms such as weakness are difficult to date. Fourth, most of the case reports reported that subjects were not taking calcitriol when they performed 1,25 (OH)2D measurements; however, few authors did not include this information in the publication.

In conclusion, our review on all TIO cases published until now aims to help clinicians to define the clinical, biochemical and radiological profile of this rare condition. By including only cases with a localized tumor and serum phosphate report, we were able to draw conclusions on the significance of clinical examination, bone evaluation and

tumor size, hitherto not reported. From our findings we can conclude that there is still a considerable diagnostic delay, despite increased knowledge of TIO and improved imaging techniques, resulting in metabolic disturbances and skeletal impairment. A thorough clinical examination can point to the causative tumor. Moreover, we found that FGF23 was related to tumor size, a finding that underlines the importance of early detection of the causative tumor followed by its removal.

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Competing interests
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Figure 3 was created with BioRender.com

Supplementary data can be accessed through the following online resources
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9474374/
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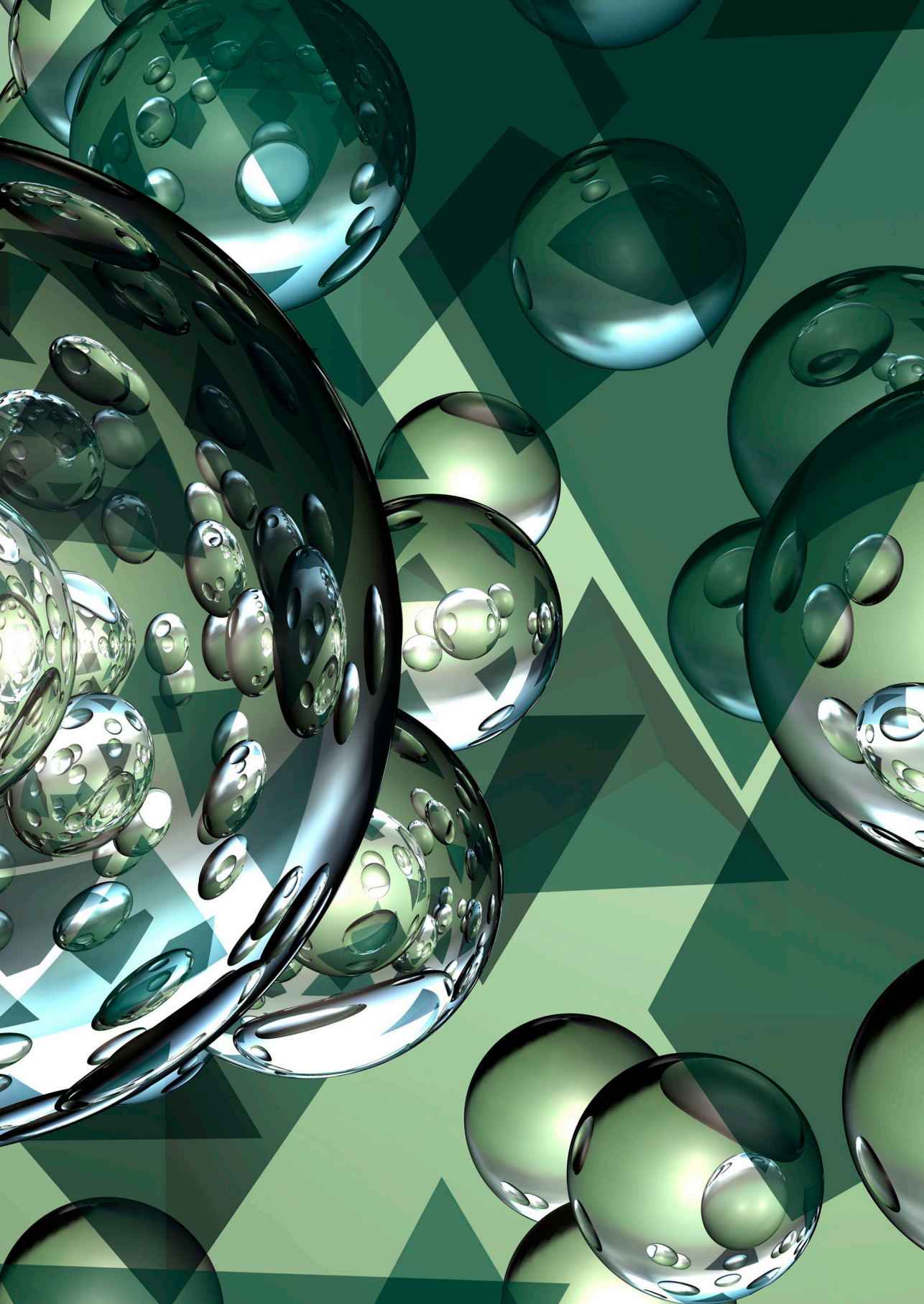

Cortisol and phosphate homeostasis: Cushing's syndrome is associated with reversible hypophosphatemia

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ABSTRACT

Objectives

The influence of hypercortisolism on phosphate homeostasis is relatively unknown. A few previous studies have reported on patients with Cushing's syndrome (CS) with hypophosphatemia in whom serum phosphate normalized after initiation of treatment for CS. We aimed to investigate the prevalence of hypophosphatemia in CS, the association between the degree of hypercortisolism and serum phosphate and the change in serum phosphate after remission of CS. We compared the prevalence of hypophosphatemia in CS with the prevalence in the population-based Rotterdam Study (RS).

Methods

Patients diagnosed with CS and treated at the Department of Endocrinology of Erasmus MC in the period of 2002-2020 were included and data was collected on age at diagnosis, sex, serum phosphate, calcium and potassium levels, kidney function and BMI. Using multivariate linear regression, we analyzed the association between 24h urinary free cortisol excretion (UFC) and serum phosphate. Changes in serum phosphate and covariates were tested with a repeated measurement ANOVA, using mean levels of laboratory values for the periods before remission, and 0-14 days and 15-180 days after remission.

Results

Hypophosphatemia before treatment was present in 16% of the 99 CS patients with data on serum phosphate, 24h UFC and covariates. In comparison, the prevalence of hypophosphatemia in RS was 2.0-4.2%. Linear regression showed a negative association between the level of UFC and serum phosphate at diagnosis, which remained significant after adjusting for covariates [β -0.002 (95%CI -0.004; -0.0004), p=0.021]. A subset of 24 patients had additional phosphate measurements at 0-14 days and 15-180 days after remission. In this subgroup, serum phosphate significantly increased from 1.03 ± 0.17 mmol/L prior to remission to 1.22 ± 0.25 mmol/L 15-180 days after remission (p = 0.008). BMI decreased after remission [-1.1 kg/m2, (95%CI -2.09 to -0.07), p=0.037]. Other covariates did not show an equivalent change over time.

Conclusion

In this retrospective study, we found that 16% of patients with CS had hypophosphatemia. Moreover, serum phosphate was related to the level of cortisoluria and increased after remission of CS. Potential underlying mechanisms related to urinary phosphate excretion and possibly involving FGF23, BMI and parathyroid hormone levels should be further explored.

Keywords
Cushing's syndrome, cortisol, hypercortisolism, phosphate, hypophosphatemia, glucocorticoids

INTRODUCTION

Cushing's syndrome (CS) results from chronic exposure to either endogenous or exogenous excess of cortisol[1]. A well-known complication of hypercortisolism in CS is glucocorticoid-induced osteoporosis (GOP)[2,3]. GOP is thought to be the result of a combination of decreased intestinal calcium absorption and renal calcium resorption, increased bone resorption, decreased bone formation and muscle wasting. Consequently, biochemical remission of Cushing's syndrome results in an increase in bone mineral density[3]. Recently, it has been suggested that treatment with glucocorticoids could also affect phosphate homeostasis and even induce hypophosphatemia due to increased urinary phosphate excretion[4,5]. Among drugs that are associated with hypophosphatemia, glucocorticoids have been suggested to be among the most common pharmacological agents associated with profound hypophosphatemia in hospitalized patients[6]. Similarly, some case reports have described hypophosphatemia in patients with CS[7-9]. After treatment for CS, normalization of serum phosphate levels has been reported after two weeks and can take up to one year[7-9]. Findling et al. (1982) reported seven patients with CS who went in remission after treatment. One year after remission, they reported a significant increase in tubular reabsorption of phosphate, a reduction in daily urinary calcium excretion, a decrease in immunoreactive parathyroid hormone (PTH) and a decrease in 1,25 dihydroxy vitamin D ($1,25(OH)_2D$)[9]. Similar to glucocorticoid use, it has been hypothesized that hypercortisolism in CS can induce hypophosphatemia by increasing urinary phosphate excretion or by inhibiting intestinal phosphate absorption. This process may be mediated by Fibroblast Growth Factor 23 (FGF23)[8,10,11]. Indeed, Delucchi et al. reported an association between sustained glucocorticoid treatment and increased intact FGF23 levels in pediatric renal transplant patients[12].

Phosphate is important for energy metabolism, cell signaling and oxygen transport. It is also a component of DNA and RNA, and it is critical for skeletal development and bone mineralization[13,14]. Most of phosphate in the human body is stored in bone, the remainder is localized in soft tissue[15]. Phosphate deficiency can cause a variety of clinical problems such as muscle weakness, rickets in children and osteomalacia in adults[16]. Phosphate homeostasis is regulated by several factors of which PTH, 1,25 dihydroxy vitamin D and FGF23 appear to be the most important[15,17]. Whereas knowledge of the role of phosphate and phosphate homeostasis is increasing, little is known about the

relation between cortisol, and specifically Cushing's syndrome (CS) and phosphate homeostasis.

The prevalence of hypophosphatemia in CS is currently unknown and the changes in phosphate concentrations after treatment for CS have only been studied in very small patient groups. Moreover, the role of potential confounders of phosphate homeostasis, such as BMI and kidney function, have not been adequately explored yet. In this study, we aim to evaluate the prevalence of hypophosphatemia in CS, the association between the level of urinary free cortisol excretion (UFC), as a marker of the degree of hypercortisolism, and serum phosphate concentrations; the role of potential confounders and the change in serum phosphate levels after remission of CS. We compare the prevalence of hypophosphatemia in CS to the prevalence in a population-based cohort study of males and females.

MATERIALS AND METHODS

Patients

This retrospective study included patients from the endocrinology department of the Erasmus University Medical Center, Rotterdam, the Netherlands, who were diagnosed with endogenous CS in the period 2002-2020. A diagnosis of CS was made based on three screening tests: late night salivary cortisol concentration, 24h UFC and the 1 mg overnight dexamethasone suppression test [1]. In patients with adrenocorticotropin hormone (ACTH) dependent CS, a pituitary-dependent cause was differentiated from an ectopic cause by bilateral inferior petrosal sinus sampling in case of a non-visible adenoma on MRI or an adenoma less than 6 mm. In patients with ACTH-independent CS, CT or MRI was performed to image the adrenal glands. To study the prevalence of hypophosphatemia and the association between 24h UFC and serum phosphate concentration, we included patients for whom serum phosphate measurements were available that were taken after diagnosis and before remission. A total of 99 patients had complete data on serum phosphate, UFC and covariates before remission and they were included to study the prevalence of hypophosphatemia and the association between UFC and serum phosphate. In addition, in the subset of this population in whom serum phosphate had also been measured after remission of CS, we studied the effect of treatment of CS on serum phosphate concentration. For 24 patients with serum phosphate measurements at time of diagnosis, serum phosphate measurements and covariates were available postoperatively and within 180 days after remission. Lastly, we determined the difference in serum phosphate concentration between the time of diagnosis and more than 180 days after remission. For this analysis we included 45 patients with a serum phosphate measurement that was taken at time of diagnosis and a serum phosphate measurement taken more than 180

days, but less than 3 years, after remission, when the patient either used hydrocortisone at a physiological dosage or supplementation had stopped. We repeated this analysis in 30 patients with additional information on covariates.

We compared the prevalence of hypophosphatemia in CS to the prevalence in the Rotterdam Study (RS). RS is a population-based study of males and females aged 40 or more from the district Ommoord in Rotterdam, the Netherlands. The rationale and design have been described in more detail elsewhere[18]. This study is ongoing since 1990 and is now composed of four cohorts, named RS-I, RS-II, RS-III and RS-IV (initiated in 1990, 2000, 2005 and 2017). The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. A total of 5,182 participants from RS-I, 2,511 from RS-II and 3,435 from RS-III with information on serum phosphate concentration were included to study the prevalence of hypophosphatemia in RS.

Methods

Serum samples from patients were analyzed as part of standard care for CS, at the department of clinical chemistry of Erasmus MC. Prior to 2013, 24h UFC was measured with a chemiluminescence immunoassay using unextracted urine (Immulite XPi, Siemens AG, Munich, Germany). The upper limit of normal (ULN) of this assay was 850 nmol/24h. From 2013 onwards, UFC was measured using liquid chromatography / tandem mass spectrometry (LC/MSMS, Waters Xevo-TQ-S, Milford, MA). The ULN of this assay is 133 nmol/24h. Hypercortisolism was defined as cortisoluria higher than the ULN of 24 hour UFC. For the purpose of harmonisation for this study, the level of cortisoluria was defined as the times of ULN (xULN) of 24 hour UFC. Data on age, sex, cause of CS, level of cortisoluria at time of diagnosis, serum phosphate and Cushing related treatment were collected from the medical files. Furthermore, we collected data on serum creatinine, total calcium, potassium, body mass index (BMI), proton-pump inhibitors (PPI) use, thiazide and loop diuretics use, as these variables have been associated with phosphate homeostasis. BMI (kg/m^2) was estimated from weight and height measured at clinical presentation. To calculate the estimated glomerular filtration rate (eGFR), the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was applied[19]. Hypophosphatemia was defined as a serum phosphate concentration below 0.80 mmol/L (normal range: 0.80-1.40 mmol/L).

In the subset of the population with serum phosphate measurements after remission of CS, the treatment modalities leading up to remission varied. In this population, the date

of remission was defined as follows: the date of biadrenalectomy or adrenalectomy; the date of removal of the ACTH producing tumor; the date of the transsphenoidal hypophysectomy resulting in remission and the date when cortisoluria was less than ULN in 24 hour urine in medically or radiologically treated patients.

Statistical analysis

The associations between 24h UFC and serum phosphate were examined through linear regression models, with the serum phosphate measurement that was taken nearest to the date of diagnosis modeled as the dependent variable and xULN of 24 hour UFC modeled as the independent variable. Analyses were adjusted for serum potassium, eGFR, total calcium, BMI, smoking and use of loop diuretics, thiazide diuretics and PPIs. To analyze the difference between mean serum phosphate before remission and several time periods after remission, we applied a repeated measures ANOVA. Measurements of serum phosphate are not part of standard care for CS[20]. Therefore, it was expected that serum phosphate had been measured at different time points and there would be missing data. To study the change in serum phosphate postoperatively and after several months, mean serum phosphate levels were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission and a repeated measures ANOVA was performed. Normality was assessed using Shapiro-Wilk's test. Analyses were repeated after exclusion of any outliers in the data. Sphericity was tested with Mauchly's test of sphericity. In the models chosen for statistical analysis, it was not possible to adjust for covariates. Therefore, any change in total calcium, potassium, eGFR and BMI was studied by comparing the means before and after remission using the statistical approach as described above. Covariates that do not show a change after remission are considered to have little or no effect on any change in serum phosphate concentrations that may be observed.

To determine the change in serum phosphate concentration in patients with a serum phosphate measurement taken at the time of diagnosis and more than 180 days after remission, we applied a paired student T-test. For this analysis we included the serum phosphate measurement that was taken nearest to the date of diagnosis and the first serum phosphate measurement that was taken more than 180 days, but less than 3 years, after remission, when the patient either used hydrocortisone at a physiological dosage or supplementation had stopped. A hydrocortisone dosage of 10 milligram in the morning, 5 milligram in the afternoon and 5 milligram in the evening was classified as physiological. We chose a cut-off of 3 years because we consider this time period to be reasonably unaffected by change due to other factors such as ageing[21].

Lastly, as a sensitivity analysis, we tested differences in serum phosphate, cortisoluria, serum calcium, potassium, eGFR, BMI and diuretics and PPI use between patients with

and without hypophosphatemia and between patients with ACTH producing pituitary adenomas and ectopic ACTH production using chi-square and Mann-Whitney U tests. Results are presented as mean ± SD, except where otherwise indicated. A p-value less than 0.05 was considered statistically significant. All analyses were performed with IBM SPSS software, version 25 (SPSS, Chicago, IL) and R version 3.6.1 (Vienna, Austria). The medical ethical committee of the Erasmus MC approved this study.

RESULTS

The general characteristics of the study population (N=99) and of the subset of the population with serum phosphate measurements at 0-14 days and 15-180 days after remission (N=24) are depicted in **Table 1**. In the total population, 73.7% was female and the mean ± SD age at diagnosis was 46.4 ± 13.5 years. An ACTH producing pituitary adenoma was diagnosed in 74.7% of patients, ectopic ACTH production was diagnosed in 23.2% of patients and 2.0% had adrenal CS. In the subset of the population with measurements at 0-14 days and 15-180 days after remission (N=24), 67% was female and the mean age at diagnosis was 50.3 ± 12.8 years. Of these 24 patients, 62.5% was diagnosed with an ACTH producing pituitary adenoma and 37.5% was diagnosed with ectopic ACTH production.

Prevalence of hypophosphatemia

In the total population of CS patients, serum phosphate at time of diagnosis was 1.01 mmol/L ± 0.21. 16% of these patients had hypophosphatemia. In RS-I of the Rotterdam Study (n=5,182), 61.4% was female, mean ± SD age was 70.3 years ± 9.0, mean serum phosphate level was 1.19 mmol/L ± 0.20 and hypophosphatemia was present in 2.0% of the population. In RS-II (n=2,511), 54.6% was female, mean ± SD age was 64.7 ± 7.8, mean serum phosphate level was 1.08 mmol/L ± 0.16 and hypophosphatemia was present in 4.2% of the population. In RS-III (n=3,435), 56.4% of patients was female, mean ± SD age was 57.1 ± 6.8, mean serum phosphate level was 1.12 mmol/L ± 0.17 and hypophosphatemia was present in 2.9% of the population.

Association between the level of cortisoluria and serum phosphate at time of diagnosis

Linear regression analyses showed a significant inverse association between serum phosphate at time of diagnosis and xULN of 24h UFC (β (95% CI): β= -0.003 (-0.005 to -0.002), p<0.001), which remained significant after adjustment for serum total calcium, potassium, eGFR, BMI, smoking, use of loop diuretics, thiazide diuretics and PPIs (β (95% CI): β= -0.002 (-0.004 to -0.0004), p=0.021). Additional adjustment for age and sex did not change results (unpublished data).

Table 1. General characteristics of the study population at time of diagnosis

	All	With 0-14 and 15-180 day measurements
N (%)	99	24
Age at diagnosis, years	46.4 (13.5)	50.3 (12.8)
Female (%)	73 (73.7%)	16 (67%)
Phosphate, mmol/L	1.01 (0.21)	1.04 (0.19)
Hypophosphatemia (%)	16 (16.2%)	2 (8.3%)
Cortisoluria, xULN UFC median (min, max)	2.6 (0.5, 144.3)	3.9 (0.6, 89.7)
Calcium, mmol/L	2.31 (0.21)	2.27 (0.18)
Potassium, mmol/L	4.0 (0.6)	3.9 (0.7)
eGFR, mL/min/1.73m²	97.7 (20.1)	100.6 (18.6)
BMI, kg/m²	29.0 (6.7)	28.9 (7.5)
Thiazide diuretics use	20 (20.2%)	4 (16.7%)
Loop diuretics use	4 (4.0%)	3 (12.5%)
PPI use	24 (23.3%)	5 (20.8%)
Current smoking	22 (21.4%)	5 (20.8%)
Cause of hypercortisolism		
– ACTH producing pituitary adenoma (%)	74 (74.7%)	15 (62.5%)
– Ectopic ACTH production (%)	23 (23.2%)	9 (37.5%)
– Adrenal CS	2 (2.0%)	-
Treatment		
– No remission (%)	8 (8.1%)	-
– Hypofysectomy(%)	22 (22.2%)	4 (16.7%)
– Medication(%)	28 (28.3%)	5 (20.8%)
– Bilateral adrenalectomy(%)	28 (28.3%)	13 (54.2%)
– Adrenalectomy	2 (2.0%)	-
– Carcinoid resection(%)	4 (4.0%)	-
– Radiation therapy(%)	6 (6.1%)	1 (4.2%)
– Unknown (%)	1 (1.0%)	-

Results are presented as mean (standard error) for continuous variables and count (percentages) for categorical variables, unless otherwise stated. BMI, body mass index; CS, Cushing's syndrome; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

Change in serum phosphate after remission

In the group of 24 patients with serum phosphate measurements after remission, mean phosphate was 1.03 ± 0.17 before remission, 1.11 ± 0.30 mmol/L at 0-14 days and 1.22 ± 0.25 mmol/L at 15-180 days after remission **Figure 1** depicts the box and whisker plots with the medians and interquartile range for the different time points. In this group, 8% had hypophosphatemia at time of diagnosis. A repeated-measures ANOVA showed that the mean phosphate levels were statistically significantly different between the different time points before and after remission F(2, 46) = 4,765, p = 0.013. A post hoc test using Bonferroni correction showed a substantial increase in serum phosphate from 1.03 mmol/L prior to remission to 1.22 mmol/L 180 days after remission, a significant increase of 0.19 (95%CI 0.04 to 0.33) mmol/L, p = 0.008 (**Figure 2**). Analysis was repeated after exclusion of outliers of serum phosphate and yielded similar results.

Next, we determined the difference in serum phosphate concentration between the time of diagnosis and more than 180 days after remission. In this group of 45 patients, serum phosphate increased significantly from 1.02 ± 0.18 mmol/L at time of diagnosis to 1.12 ± 0.25 mmol/L at >180 days after remission, a significant increase of 0.09 mmol/L (95%CI 0.02 to 0.17, p=0.019). Results were similar when restricting the analysis to patients who also had serum total calcium, eGFR, potassium and BMI measured more than 180 days after remission: increase of 0.11 mmol/L (95%CI 0.001 to 0.21), p=0.051, N=30).

Figure 1. Box and whisker plots illustrating serum phosphate concentrations before remission, 0-14 days after remission and 15-180 days after remission. Boxes include medians and interquartile range. Whiskers extend 1.5 times the interquartile range.

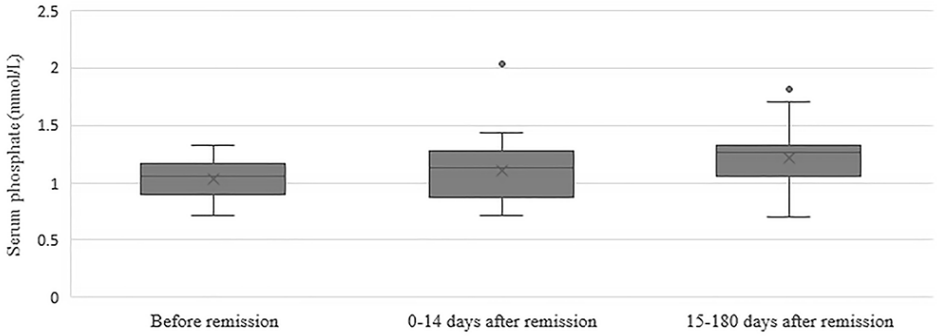
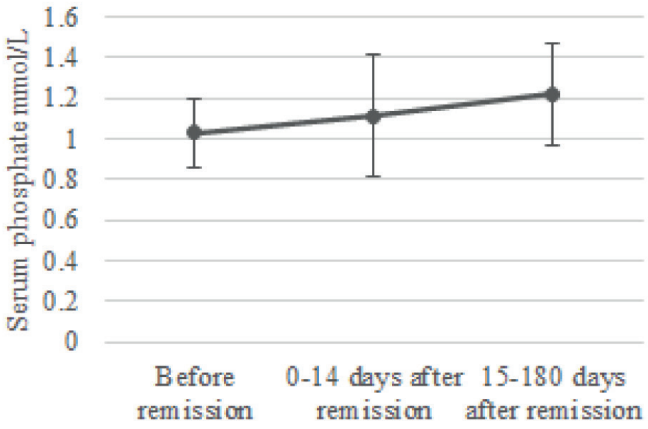


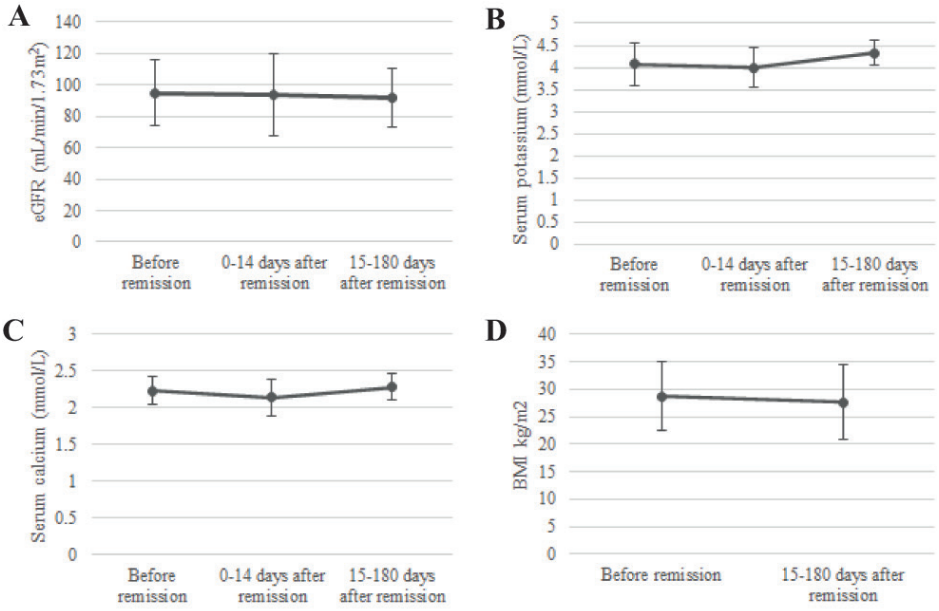
Figure 2. Change in serum phosphate concentration after remission. Mean serum phosphate levels and standard deviation were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission



Changes in covariates after remission

In the group op 24 patients with phosphate measurements 0-14 days and 15-180 days after remission, no change was observed in eGFR after remission of CS. There was a slight increase in serum potassium concentration from 3.99 ± 0.45 mmol/L 0-14 days after remission to 4.33 ± 0.28 mmol/L 15-180 days after remission, a significant increase of 0.34 (95%CI, 0.07 to 0.61) mmol/L, p = 0.009. Moreover, serum total calcium increased from 2.14 ± 0.25 mmol/L at 0-14 days after remission (p=0.046) to 2.28 ± 0.18 mmol/L at 15-180 days after remission (p=0.034). Lastly, we observed a significant decrease in BMI when comparing BMI at 15-180 days after remission with BMI before remission (paired t-test:-1.1 (95%CI-2.09 to-0.07), p=0.037) (**Figure 3**).

Figure 3. Change in eGFR (A), serum potassium (B), serum calcium (C) and BMI (D) after remission. Means and standard deviations were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission. Abbreviations: eGFR, estimated glomerular filtration rate.



In the group of 30 patients with serum phosphate, total calcium, potassium, eGFR and BMI measurements taken at time of diagnosis and more than 180 days after remission, no change was observed in serum calcium. Interestingly, eGFR decreased from 98.83 ± 19.66 mL/min/1.73m^2 to 83.30 ± 24.39 mL/min/1.73m^2, a significant decrease of 15.53 mL/min/1.73m^2 (95%CI 8.07 to 22.97, p-value <0.001), while serum potassium increased from 4.11 ± 0.57 mmol/L to 4.46 ± 0.38 mmol/L, a significant increase of 0.35 (95%CI 0.05 to 0.64, p-value=0.022). BMI decreased from 30.2 ± 1.4 to 28.2 ± 1.5, a significant

decrease of 2.0 (95%CI-3.0 to-0.9, p-value=0.001).

Lastly, differences between patients with hypophosphatemia and without hypophosphatemia and between patients with ACTH producing pituitary adenomas and ectopic ACTH production were tested using chi-square and Mann-Whitney U tests. Differences between patients with hypophosphatemia and without hypophosphatemia are depicted in **Table 2**. Here, xULN of 24h UFC was higher in patients with hypophosphatemia than in patients without hypophosphatemia (p=0.024). Patients with hypophosphatemia had lower serum calcium levels (p<0.001) and were more likely to have CS from ectopic ACTH production than patients without hypophosphatemia. Differences between patients with ACTH producing pituitary adenomas and with ectopic ACTH production are depicted in **Table 3**. Patients with CS from ectopic ACTH production were older (p=0.031), had lower phosphate concentration at time of diagnosis (p=0.004), were more likely to have hypophosphatemia (p=0.023), had higher xULN of 24h UFC (p<0.001) and had lower potassium concentrations (p<0.001) than patients with CS from ACTH producing pituitary adenomas.

Table 2. Differences between patients with hypophosphatemia and with normal phosphate concentration before remission

	Hypophosphatemia	**Normal phosphate**	***P***
N (%)	16	83	
Age at diagnosis in years	49.9 (19.7)	46.5 (19.5)	0.849
Female (%)	13 (81.3%)	60 (72.3%)	0.550
Phosphate, mmol/L	0.68 (0.13)	1.06 (0.17)	<0.001
Cortisoluria, xULN UFC	5.6 (53.9)	2.6 (3.0)	0.022
Calcium, mmol/L	2.17 (0.37)	2.33 (0.21)	0.003
Potassium, mmol/L	4.0 (0.9)	4.1 (0.7)	0.362
eGFR, mL/min/1.73m²	101.7 (29.5)	100.1 (26.6)	0.680
BMI, kg/m²	26.8 (6.6)	27.6 (9.4)	0.356
Thiazide diuretics use	0	20 (24.1%)	0.037
Loop diuretics use	2 (12.5)	2 (2.4%)	0.121
PPI use	6 (37.5%)	17 (20.5%)	0.126
Current smoking	3 (18.8%)	17 (20.5%)	0.590
Cause of hypercortisolism			
– ACTH producing pituitary adenoma (%)	8 (50.0%)	66 (79.5%)	0.044
– Ectopic ACTH production (%)	7 (43.8%)	16 (19.3%)	
– Adrenal CS (%)	1 (6.3%)	1 (1.2%)	

Results are presented as median (interquartile range) for continuous variables and count (percentages) for categorical variables. ACTH, adrenocorticotropin hormone; BMI, body mass index; CS, Cushing's syndrome; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

Table 3 Differences between patients with an ACTH producing pituitary adenoma and ectopic ACTH production before remission

	ACTH producing pituitary adenoma	ectopic ACTH production	*P*
N (%)	74	23	
Age at diagnosis in years	44.6 (17.7)	54.6 (22.6)	0.031
Female (%)	55 (74.3%)	16 (69.6%)	0.788
Phosphate, mmol/L	1.06 (0.21)	0.92 (0.30)	0.004
Hypophosphatemia	8 (10.8%)	7 (30.4%)	0.042
Cortisoluria, xULN UFC	2.3 (2.1)	19.1 (42.1)	<0.001
Calcium, mmol/L	2.35 (0.20)	2.22 (0.24)	0.005
Potassium, mmol/L	4.1 (0.6)	3.7 (1.1)	<0.001
eGFR, mL/min/1.73m^2	99.4 (27.9)	106.9 (20.9)	0.031
BMI, kg/m^2	28.2 (9.0)	24.5 (4.7)	0.004
Thiazide diuretics use	20 (27.0%)	0	0.003
Loop diuretics use	3 (4.1%)	1 (4.3%)	1.0
PPI use	16 (21.6%)	7 (30.4%)	0.408
Current smoking	17 (23.0%)	3 (13.0%)	0.387

Results are presented as median (interquartile range) for continuous variables and count (percentages) for categorical variables. Abbreviations: ACTH, adrenocorticotropin hormone; BMI, body mass index; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

DISCUSSION

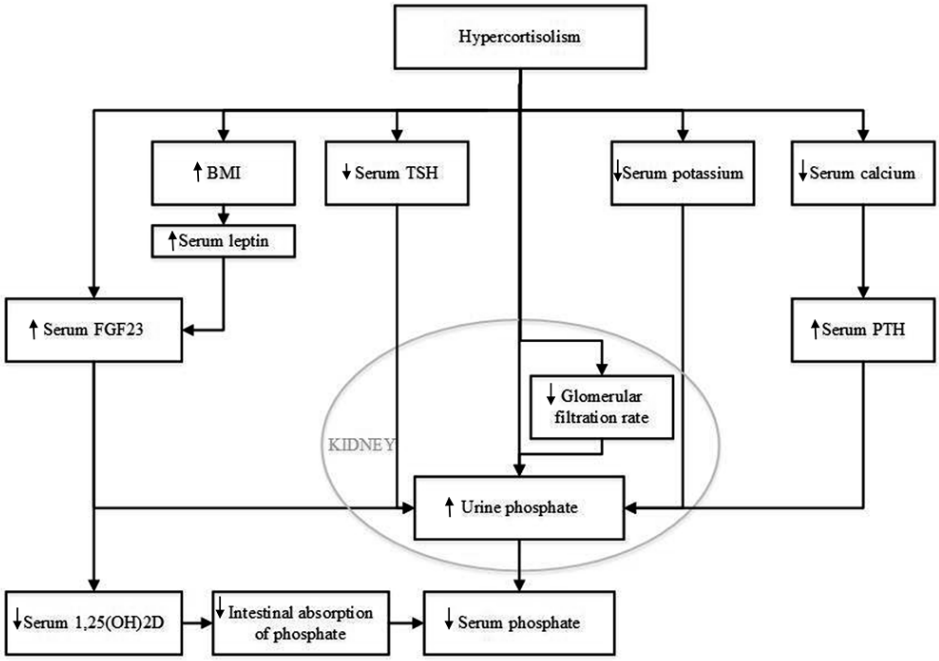
In this study we investigated the prevalence of hypophosphatemia in CS, and the change in serum phosphate concentration and potential confounders of phosphate homeostasis after remission of CS. In addition, we explored the association between UFC and serum phosphate before remission. Data from our study showed that hypophosphatemia was present in up to 16% of our patients with active CS. The prevalence of hypophosphatemia in these patients is four to six times higher than in participants from a population-based cohort study. We also found that serum phosphate increases after remission, which also suggests that hypercortisolism affects serum phosphate concentration. The higher degree of hypercortisolism found in hypophosphatemic patients and the inverse association between the UFC level and serum phosphate concentration indicate modulatory effects of cortisol on phosphate homeostasis.

Our results indicate that hypercortisolism in CS affects serum phosphate even to the extent of causing hypophosphatemia. Hypophosphatemia can cause multiple symptoms such as fatigue and muscle weakness, which are complaints that are commonly reported by CS patients. Nearly 60% of patients with Cushing's syndrome have muscle weakness[22]. Glucocorticoid induced myopathy is caused by an altered protein metabolism, resulting in muscle atrophy and muscle protein catabolism[22]. In addition, it has been suggested

that hypophosphatemia causes a decrease in muscle ATP synthesis[23]. Consequently, hypophosphatemia may worsen muscle weakness in patients with CS and may also contribute to the development of glucocorticoid-induced low bone mineral density and fractures by causing osteomalacia. As can be expected, patients with CS based on ectopic ACTH production had higher UFC levels than patients with CS due to ACTH producing pituitary adenomas[24], and were in turn more likely to develop hypophosphatemia.

Our findings are in line with and extend previous reported cases of hypophosphatemia in CS, in whom remission of CS resulted in normalization of serum phosphate[7,8]. Similarly, Findling et al. reported previously an increase in serum phosphate concentration after treatment of ACTH-dependent CS in 7 patients. However, the pathophysiological mechanism(s) for these changes in serum phosphate concentrations is largely unknown. Previous studies have suggested that glucocorticoids may reduce intestinal absorption of phosphate and increase renal phosphate excretion[4,10]. Indeed, Findling et al. observed an increase in the tubular reabsorption rate of phosphate (TRP) after treatment for CS.

Figure 4 Potential mechanisms that could explain the effect of hypercortisolism on serum phosphate concentration. Abbreviations: BMI, body mass index; FGF23, Fibroblast Growth Factor 23; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone; 1,25(OH)2D, 1,25 dihydroxy vitamin D.



There are several potential hypothetical mechanisms that could explain the effect of glucocorticoids on serum phosphate concentration. These are summarized in **Figure 4**. One pathophysiological mechanism relates to FGF23, which is mainly expressed and secreted by osteocytes and osteoblasts[8,10,11]. Expression of FGF23 is regulated by serum phosphate concentration. FGF23 regulates serum phosphate by e.g., increasing urinary phosphate excretion, but the role of glucocorticoids in FGF23 regulation is still unclear. Delucchi et al. (2019) reported an association between sustained glucocorticoid treatment and increased intact FGF23 levels in pediatric renal transplant patients[12]. The same group reported an increase in bone FGF23 protein abundance and in FGF23 expression in MG53 cells, a human osteosarcoma cell line, when incubated with dexamethasone[12]. In contrast, Feger et al. (2021) reported a downregulation of FGF23 transcription and protein synthesis in UMR106 rat osteoblast-like cells and MC3T3-E1 cells after incubation with dexamethasone or prednisolone. Similarly, injection of dexamethasone or prednisolone in mice lead to a decrease of serum C-terminal and intact FGF23 concentration and bone FGF23 mRNA expression, but, strikingly, also to increased renal phosphate excretion and decreased serum phosphate concentration, without affecting PTH[25]. The authors state that their findings could be explained by the inhibitory effect of dexamethasone on membrane expression of sodium-dependent phosphate transporters in the kidney, resulting in increased renal phosphate excretion, as was previously reported[26]. FGF23 is not routinely measured in patients with CS but Endo et al. (2008) reported a patient with hypophosphatemia due to ectopic ACTH production whose active FGF23 concentration was below the mean value previously found in healthy adults[11]. We also recently observed normal C-terminal FGF23 levels in a patient who was diagnosed with hypophosphatemia and adrenal CS (unpublished observations). In this patient, serum phosphate concentration also recovered after adrenalectomy. These findings would support the hypothesis that the effect of glucocorticoids on serum phosphate concentration is independent of FGF23 and thus might be related to an effect of GCs on the sodium-dependent phosphate transporters. However there is clearly a need for larger studies on intact and C-terminal FGF23 before and after treatment of CS.

A second pathophysiological mechanism might relate to BMI. The majority of CS patients develop obesity[1,27]. Although the treatment of CS has been shown to lower BMI, patients treated for CS maintain a higher BMI than controls matched by sex and age[28,29]. Indeed, our study showed a decrease in BMI after treatment for CS. Previous literature has shown that BMI and serum phosphate levels are inversely associated[30,31]. Moreover, we recently observed evidence for a causal effect of BMI on serum phosphate using a Mendelian Randomisation approach (unpublished data). There are several theories on the pathophysiological mechanism behind this effect. A higher BMI is associated with lower 25-hydroxyvitamin D levels [32], which in turn could result in lower levels of

$1,25(OH)_2D$ leading to impaired phosphate absorption from the intestine. FGF23 may also play a role in adiposity associated decreases in serum phosphate as adiposity has also been associated with FGF23. Leptin, which has been shown to function as a FGF23 secretagogue, is strongly related to adiposity[33-35]. Put differently, the change in serum phosphate levels after treatment for CS may be, at least in part, due to the decrease in BMI.

A third potential mechanism may involve kidney function. An important consequence of chronic hypercortisolism is the increased risk for cardiovascular complications, including atherosclerotic vascular damage[28]. In a case-control study in 18 patients, Haentjes et al. showed that patients with Cushing's disease have a decreased glomerular filtration rate compared to controls[36]. Early stages of chronic kidney disease are associated with increased FGF23 levels and hyperphosphaturia[37]. However, in these early stages of chronic kidney disease, serum phosphate levels are still maintained in the normal range. Hence, this would not explain why CS patients are more likely to develop hypophosphatemia.. We did not observe a change in estimated glomerular filtration when we compared eGFR at time of diagnosis with 0-14 days and 15-180 days after remission. In contrast, we observed a decline in eGFR more than 180 days after remission.

A fourth possible pathophysiological mechanism that we considered involves serum potassium. Rat studies have shown that a potassium deficiency can result in phosphaturia[38]. Similarly, in humans, potassium supplementation leads to a decrease in FGF23 and an increase in serum phosphate levels[39]. Hypokalaemia can occur in any patient with CS[40]. Due to hypercortisolism, the 11β-hydroxysteroid dehydrogenase type 2 enzyme, which converts cortisol into cortisone, can get saturated. Saturation of this enzyme results in activation of mineralocorticoid receptors, which results in increased renal excretion of potassium. Although we observed a slight increase in serum potassium concentration from 0-14 days after remission to 15-180 days after remission, we did not find a significant difference when comparing serum potassium before remission with serum potassium after remission.

A fifth potential pathophysiological mechanism involves serum calcium. Both serum calcium and serum phosphate levels are regulated by $1,25(OH)_2D$ and PTH. It has been postulated that glucocorticoids inhibit calcium absorption from the intestinal tract, but this effect remains controversial[41]. In the case series of Findling et al, serum calcium did not change, but there was a reduction observed in urinary calcium excretion after treatment for CS. Interestingly, we observed a decrease in serum calcium levels at 0-14 days after remission compared to before remission. This decrease however was not seen for the period of 15-180 days after remission. In theory it is still possible that increased urinary calcium excretion combined with decreased intestinal absorption during active

CS results in secondary hyperparathyroidism with an increase in urinary P excretion. Unfortunately, serum PTH levels were not measured in our patients because serum calcium was normal.

Other hypothetical mechanisms that could be considered include the role of hypothalamic-pituitary axes such as the hypothalamic-pituitary-thyroid axis. Thyroid-stimulating hormone and thyroid hormone can be influenced by glucocorticoid excess which may affect serum phosphate homeostasis[41-43]. Most of our patients had ACTH dependent Cushing's syndrome. There is evidence that ACTH influences bone mass[44,45], but a direct effect of ACTH on phosphate homeostasis remains to be elucidated.

This study has several limitations. A major limitation is the retrospective nature of the study and the considerable number of missing data. Because serum phosphate was not measured at set time points, we calculated mean serum phosphate levels. We can assume that this will negatively affect the variance of phosphate over time. To draw conclusions on the course of the phosphate levels over time we calculated several time points, including 0-14 days and 15-180 days after remission. It is not known at what time during the day the blood samples were drawn, which could affect serum phosphate levels[46]. Finally, serum FGF23, $1,25(OH)_2$ D, PTH nor urinary phosphate concentrations were available to us.

In conclusion, we showed that hypophosphatemia can occur in up to 16% of patients with CS, that serum phosphate concentration is related to the degree of hypercortisolism and that remission of CS results in an increase in serum phosphate. Effects were stronger in patients with CS due to ectopic ACTH production. These results suggest that hypercortisolism in CS affects phosphate homeostasis. We postulate that hypophosphatemia in CS patients may contribute to fatigue, muscle weakness and impaired bone quality. Therefore, the effect of hypercortisolism on FGF23 and urinary phosphate excretion should be further evaluated in a prospective setting and all patients with CS should be evaluated for hypophosphatemia, especially when it concerns CS from ectopic ACTH production.

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Contribution to the Field
Cushing's syndrome (CS) is a result of chronic exposure to either endogenous or exogenous excess cortisol. A well-known comorbidity of hypercortisolism in CS is glucocorticoid-induced osteoporosis. This type of osteoporosis is thought to be the result of a combination of decreased intestinal calcium absorption and renal calcium resorption, increased bone resorption, decreased bone formation and increased muscle wasting. Recently, it has been suggested that treatment with glucocorticoids could also affect phosphate homeostasis and even induce hypophosphatemia due to increased urinary phosphate excretion. Similarly, several case reports have described hypophosphatemia in patients with CS, which resolved after treatment for CS. The prevalence of hypophosphatemia in CS is currently unknown and the change in serum phosphate after treatment for CS has only been studied in very small patient groups. This retrospective study showed that, before remission, hypophosphatemia can occur in up to 16% of patients with CS. The prevalence of hypophosphatemia in these patients is four to six times higher than in participants from a population-based cohort study. Moreover, serum phosphate was related to the degree of hypercortisolism in CS and serum phosphate increased after remission. These findings suggest that hypercortisolism affects serum phosphate levels, even to the extent of hypophosphatemia. Hypophosphatemia can result in fatigue and muscle weakness, which are symptoms that are commonly reported by CS patients. We make the case that hypophosphatemia in CS may contribute to fatigue, muscle weakness and impaired bone quality. The pathophysiological mechanism and the clinical consequences should be further explore

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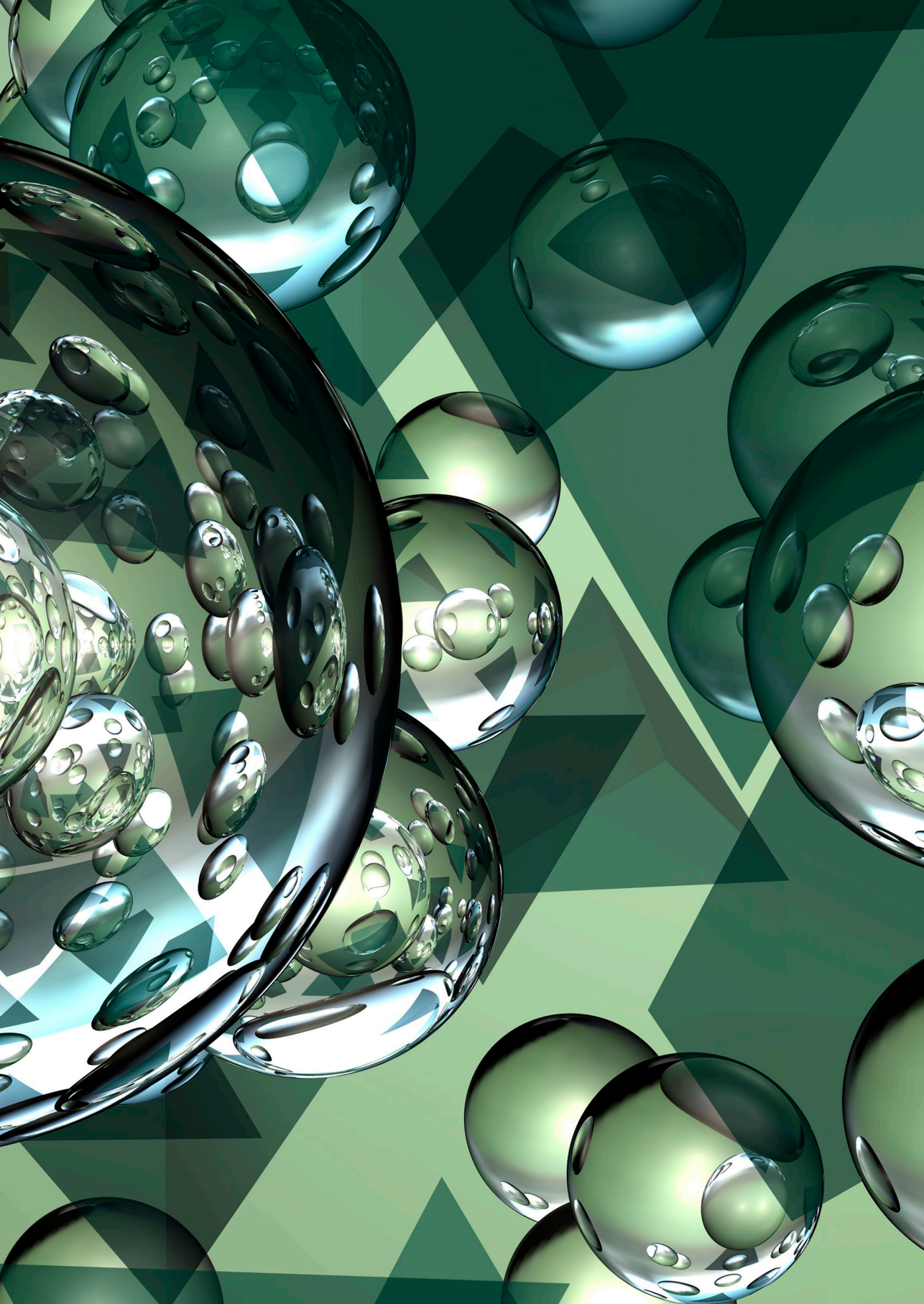

Case report: Unexplained mild hypophosphatemia and very high serum FGF23 concentrations

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8

ABSTRACT

Fibroblast growth factor (FGF)23 is one of the major regulators of phosphate homeostasis. Hypophosphatemia can lead to muscle weakness, fatigue and osteomalacia. In the setting of hypophosphatemia, serum FGF23 can be measured to differentiate between FGF23-mediated and non-FGF23-mediated renal phosphate wasting. C-terminal FGF23 (cFGF23) assays detect both cFGF23 and intact FGF23 (iFGF23). Circulating FGF23 is regulated by 1.25-dihydroxy-vitamin D, PTH, serum phosphate and serum calcium but also by e.g., iron status, inflammation, erythropoietin, and hypoxia-inducible-factor-1-α. We present the case of a 48-year-old woman with unexplained mild hypophosphatemia, very high cFGF23 and normal iFGF23. The patient proved to have an iron deficiency. Iron deficiency alters the iFGF23-to-cFGF23 ratio. After initiation of iron treatment, cFGF23 strongly decreased. This case report illustrates the limitation of cFGF23 assays and urges clinicians to be aware that cFGF23 concentrations do not necessarily reflect iFGF23 concentrations and that alternative causes for its elevation should be considered (e.g., iron deficiency).

Keywords

FGF23, phosphate, hypophosphatemia, iron, , iron deficiency

INTRODUCTION

Inorganic phosphate is important for several metabolic processes, including intracellular signal transduction and energy production, and for mineralization of the skeleton by formation of hydroxyapatite. Acute severe hypophosphatemia can lead to neurological symptoms and impaired cardiac and respiratory function. Chronic hypophosphatemia is usually characterized by muscle weakness, fatigue and the development of osteomalacia, accompanied by bone pain[1]. One of the major regulators of phosphate is fibroblast growth factor (FGF)23[2]. Recently it has been shown that FGF23 also interacts with iron metabolism and erythropoiesis[3]. The current case illustrates the interaction between FGF23 and iron status, and challenges the applicability of cFGF23 assays for the work-up of hypophosphatemia in the setting of FGF23-related disturbances.

CASE DESCRIPTION

A 48-year-old woman was referred to the nephrologist of Erasmus Medical Center in 2012 suffering from bone and joint pain, fatigue and muscle weakness since six months. Her family history was negative for bone related conditions and her physical examination did not show signs of growth retardation or bone deformities. Her medical history included asthma; microcytic hematuria; iron deficiency for which she had used iron supplementation in the past; and pangastritis in 2010. She used salbutamol and formoterol/beclometasone for her asthma, and omeprazole. At biochemical evaluation, kidney function, serum sodium, potassium and magnesium were within the reference range, but her phosphate was low, 0.71 mmol/L (normal range, 0.80 – 1.40). Albumin-adjusted calcium was 2.25 mmol/L (normal range, 2.20-2.65), 25-hydroxy-vitamin D (25OHD) was 107 nmol/L (normal range, 50-120), 1.25-dihydroxy-vitamin D ($1.25OH_2D$) was slightly increased, 197 pmol/L (normal range, 38-183) and intact parathyroid hormone (PTH) was 7.2 pmol/L (normal range, 1.4-7.3) (**Table 1**). The ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate (TMP/GFR) was 0.57 mmol/L (normal range, 0.84-1.23) and C-terminal Fibroblast Growth Factor 23 (cFGF23) (Immutopics, San Clemente, CA, USA[4]) was 234 RU/mL (normal range, <125), suggesting FGF23-related renal phosphate wasting. There were no signs of a generalized tubulopathy, e.g., glucosuria or proteinuria, and renal ultrasound showed no nephrocalcinosis or nephrolithiasis. Whole exome sequencing was performed for renal hypophosphatemic disorders and showed no mutations in genes *SLC34A1*, *SLC34A3* or *SLC9A3R1*. She was treated with oral phosphate supplementation three times daily 20 mmol resulting in repeatedly normal serum phosphate measurements, ranging between 0.86 and 1.39 mmol/L, and she was discharged from follow-up.

Six years later, in 2018, she was referred to the Bone Center of our hospital with persistent incapacitating fatigue and bone-, muscle- and joint pain. She had never suffered a fracture. Serum phosphate was 1.39 mmol/L while on phosphate supplementation, but after cessation serum phosphate decreased to 0.77 mmol/L. At this time, albumin-adjusted calcium was 2.45 mmol/L (normal range, 2.20-2.65), 25OHD was 79 nmol/L, $1.25(OH)_2D$ was 96.5 pmol/L, intact PTH was 3.9 pmol/L (normal range, 0.68-4.40). Iron status was not measured at this time, but was normal earlier that year, when serum phosphate was 0.85 mmol/L. 24h urine calcium was 6.72 mmol (normal range, 2.5-7.5 mmol/24h). Celiac disease was excluded based on absence of endomysial antibodies. Despite the fact that hypophosphatemia was mild and responded well to supplementation, TmP/GFR was repeatedly decreased, suggesting renal phosphate wasting. cFGF23, measured later that year, was 697 RU/mL (**Table 1**). The bone mineral density (BMD) assessment by dual-energy X-ray absorptiometry (DXA) revealed a T-score of -2.1 SD at the femoral neck and -2.1 SD at the lumbar spine (normal: T-score >-1 SD). Whole exome sequencing was repeated and showed no mutations in *FGF23* and *PHEX*. Our differential diagnosis included tumor-induced osteomalacia (TIO) but physical examination and CT, FDG-PET-CT and 68-Gallium-DOTATE PET-CT did not show signs of a causative tumor.

In 2019, cFGF23 had increased up to 935 RU/ml, while serum phosphate was normal or slightly decreased without supplementation. At that time, serum 25OHD was decreased (20 nmol/L) and serum alkaline phosphatase (AF) was elevated, 177 U/L (normal range, <98) as well as bone specific alkaline phosphatase, 48.9 µg/L (normal range, <14.3). A bone biopsy showed severe osteoporosis and low bone turnover but no signs of osteomalacia. We initiated cholecalciferol after which serum 25OHD and AF normalized, but serum phosphate was still decreased, 0.77 mmol/L.

In the years that followed, cFGF23 concentrations kept rising and there was no finding of TIO on imaging. Because iron deficiency can be associated with hypophosphatemia and the patient had been treated with iron supplementation for iron deficiency in the past, we measured her iron status again in 2022. At this time, her hemoglobin was 8.0 mmol/L (normal range, 7.5-9.5), mean corpuscular volume was 80 fl (normal range, 80-100), but her iron was 5.7 µmol/L (normal range, 10-30), indicating iron deficiency. Due to the discrepancy between the very high cFGF23 and normal or slightly decreased serum phosphate, we decided to measure the C-terminal and intact FGF23 (iFGF23) in our patient and in a healthy control in our research lab for Calcium and Bone Metabolism, since measurement of iFGF23 for clinical purposes was not available to us at that time. cFGF23 was 1260 RU/mL in the patient and 51 RU/mL in the healthy control (MicroVue Human FGF-23 (C-Term) EIA). iFGF23 was 36 pg/mL in our patient and 47 pg/mL in the healthy control (MicroVue Bone Human FGF-23 (Intact) EIA). After excluding gastrointestinal blood loss by performing gastroscopy and colonoscopy, we

treated the iron deficiency with ferrous fumarate 200mg three times daily. Serum iron increased to 18.5 µmol/L, serum phosphate was normal at 0.95 mmol/L without phosphate supplementation and cFGF23 decreased from 935 to 158 RU/mL. The patient was pleased that the cause for the high cFGF23 concentrations was found and that she did not have a tumor. The iron supplementation improved her fatigue but the muscle pain remained.

Table 1. Laboratory results during follow-up of the patient

Serum	2012	2018	2019	2022 (before iron supplementation)	2022 (after iron supplementation)
Phosphate (mmol/L) (normal: 0.80-1.40)	0.71	0.77	0.83	0.77	0.95
25(OH)D (nmol/L) (normal: 50-120)	107	79	20	80	79
1.25$(OH)_2$D (pmol/L) (normal: 38-183)	197	96.5	-	-	-
Alkaline phosphate (U/L) (normal: <98)			177	93	92
PTH (pmol/L) (normal 0.68-4.40)	7.2†	3.9	3.3		
cFGF23 (RU/mL) (normal <125)	234	697	935	-	158
cFGF23 (RU/mL)‡	-	-	-	1260	-
iFGF23 (pg/mL)‡	-	-	-	36	-
hemoglobin (mmol/L) (normal: 7.5-9.5)	8.2	-	7.9	8.0	9.4
MCV (fl) (normal: 80-100)	81	-	78	80	92
iron (µmol/L) (normal:10-30)	-			5.7	18.5
Ferritin (µg/L) (normal 10-140)				6	67

†normal range PTH in 2012: 1.4-7.3 pmol/L. ‡ C-terminal FGF23 and intact FGF23 were measured simultaneously in our research lab in 2022.

DISCUSSION

We describe the medical history of a 48-year old female patient with bone pain, fatigue, muscle complaints, mild hypophosphatemia and very high cFGF23 concentrations. Because cFGF23 concentrations kept rising we conducted extensive diagnostic procedures including advanced imaging, in search of a growing tumor causing tumor-induced osteomalacia (TIO), and genetic testing[5]. It turned out she had normal iFGF23 concentrations and increased cFGF23 due to iron deficiency. This case raises two questions: 1. Can elevated cFGF23 cause mild hypophosphatemia in the setting of iron

deficiency and 2. Is the use of cFGF23 assays to differentiate the between causes of hypophosphatemia appropriate in the setting of serum FGF23 disturbances such as iron deficiency.

Serum phosphate is mainly regulated by 1.25 dihydroxyvitamin D ($1.25(OH)_2D$), parathyroid hormone (PTH) and FGF23. $1.25(OH)_2D$ increases phosphate absorption from the intestine whereas PTH and FGF23 stimulate renal phosphate excretion. Both $1.25(OH)_2D$ and PTH induce bone resorption which leads to release of phosphate. FGF23 also inhibits 1α-hydroxylase, thereby decreasing the synthesis of $1.25(OH)2D$[2]. The differential diagnosis of hypophosphatemia can be divided in three major groups: hypophosphatemia from increased renal excretion, from decreased intestinal absorption or due to intracellular shift of phosphate. Increased renal excretion can be subdivided in FGF23-mediated and non-FGF23-mediated renal phosphate wasting[1,6]. Increased FGF23 concentrations can be caused by monogenetic disorders such as X-linked hypophosphatemia, or by a FGF23-producing tumor in the setting of TIO[1,5].

FGF23 is mainly produced by osteoblasts and osteocytes. Classical regulators of circulating FGF23 include $1.25(OH)_2D$, PTH, alpha-klotho, serum phosphate and serum calcium. Non-classical regulators of FGF23 include iron status, inflammation, erythropoietin, hypoxia inducible factor 1α (HIF1α), insulin and diabetes and leptin[7]. The *FGF23* gene encodes for a 251-amino acid long glycoprotein that requires post-translational O-glycosylation in order to be stabilized and phosphorylation to be cleaved[3,7]. Currently, two types of FGF23 assays exist for the quantification of circulating FGF23 concentrations in humans. The first one is the C-terminal assay, which detects both the active full-length FGF23 and the C-terminal fragments that are released after cleavage, by using antibodies that target epitopes in the C terminus. The second one is the intact FGF23 assay which only detects the presumed active full-length FGF23[3]. In the Netherlands, FGF23 concentrations are determined at the Amsterdam University Medical Center by a C-terminal FGF23 (cFGF23) assay (Immutopics, San Clemente, CA, USA) (upper limit of normal: 125 RU/mL)[4].

Edmonston *et al.* describe the application of an intact FGF23-to-C-terminal FGF23 ratio (i:cFGF23), in combination with the concomitant serum phosphate level, in discriminating between different FGF23-mediated syndromes[3]. Two types of conditions that can lead to high cFGF23 with normal or low iFGF23 concentrations, resulting in a low i:cFGF23 ratio, have been described. On the one hand there are conditions, including iron deficiency, inflammation and increased erythropoietin (EPO), that stimulate *FGF23* transcription and cleavage resulting in high cFGF23 but normal iFGF23 concentrations. On the other hand there is a condition named tumoural calcinosis, which can be caused by a mutation in N- acetylgalactosaminyltransferase 3 (*GALNT3),* resulting in increased cleavage of

iFGF23 with high cFGF23 and low iFGF23 concentrations.

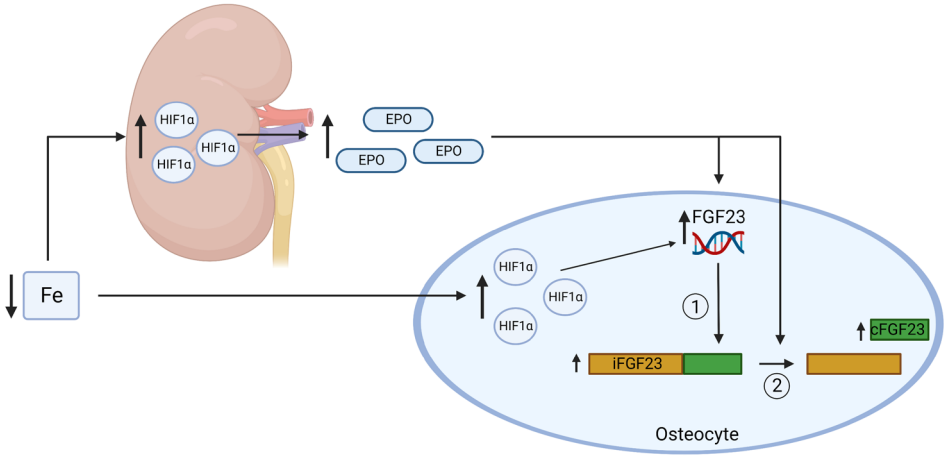
The interaction between FGF23 and iron status and erythropoiesis has been extensively studied. Induction of iron deficiency by a low iron diet in wild-type mice resulted in increased serum intact FGF23 and C-terminal FGF23 concentrations, with a corresponding decrease in 1.25(OH)2D concentrations and an increase in urinary phosphate excretion but no change in serum phosphate[8]. The role for iron in FGF23 regulation in humans was illustrated by Imel *et al.* in patients with autosomal dominant hypophosphatemic rickets (ADHR), which is caused by mutations in the *FGF23* gene leading to impaired cleavage of iFGF23[9]. In this study, low serum iron was associated with both elevated cFGF23 and iFGF23 in patients with ADHR, but only with elevated cFGF23 in healthy controls. This suggested that iron deficiency leads to increased *FGF23* expression, but increased cleavage retains homeostasis in humans without ADHR. Similarly, serum iron was found to be associated only with increased cFGF23 and not with iFGF23 in a study in healthy premenopausal women. In this study, serum phosphate correlated with iFGF23 in all women, but with cFGF23 in black women only[10]. In contrast, in a Swedish population-based cohort study of elderly men, low serum iron was found to be associated with high serum iFGF23[11].

The effect of iron status on FGF23 could be mediated by erythropoietin (EPO) and HIF1α (**Figure 1**). EPO is upregulated in the setting of iron deficiency. It stimulates red blood cell production and reduces hepcidin to release iron from stores[12]. It has been reported that EPO affects both production and cleavage of FGF23[13]. In human studies with administration of recombinant EPO, iFGF23 remains normal or increases only slightly, while the increase in cFGF23 is more pronounced[13]. HIF1α is stabilized in the setting of hypoxia or iron deficiency and activates the production of EPO. Interestingly, HIF1α may also affect FGF23 directly[8]. David *et al.* conducted a study in mice who were injected with IL-1β to mimic a pro-inflammatory state, leading to functional iron deficiency. These mice were found to have increased cFGF23, while iFGF23 remained unchanged. When IL-1β injected MC3T3-E1 osteoblast-like cells were pretreated with a HIF1α inhibitor, the effects of IL-1β on *FGF23* mRNA expression and FGF23 protein were partially blocked, indicating that HIF1α may target FGF23 transcription directly[8].

We acknowledge the limitation of our case study that the cFGF23 and iFGF23 assays used in our research lab are not meant for clinical practice. However, the iFGF23 concentration in our patient was comparable to the healthy control and it is clear that our patient had a very low i:cFGF23 ratio, which can point to untreated iron deficiency[3]. In iron deficiency, iFGF23 remains mostly unchanged, but it is possible that iFGF23 concentrations were increased at a certain point in time, causing the mild hypophosphatemia. However, other causes of mild hypophosphatemia should be

considered e.g., hyperparathyroidism, use of alcohol, and less well-known causes of hypophosphatemia such as obesity and Cushing´s disease[1,14,15]. After treatment of the iron deficiency, serum phosphate concentrations were normal but the complaints of the patient only partly resolved. The muscle pain remained, which suggests that the mild hypophosphatemia was not clinically significant and that there is another cause for the complaints than hypophosphatemia. Still, her high cFGF23 concentrations led to extensive diagnostic procedures and a significant diagnostic delay, which would not have been conducted had we known iFGF23 concentrations. Alarmingly, the fact that cFGF23 do not necessarily reflect iFGF23 concentrations is not incorporated in commonly applied diagnostic algorithms of hypophosphatemia[6]. In the Netherlands and also in other countries, hospitals only use cFGF23 assays and not iFGF23 assays, while Hartley et al. found that measurement of iFGF23 is superior to cFGF23 in making a diagnosis of FGF23-mediated hypophosphatemia[16-18].

Figure 1. Potential mechanism that could explain the effect of iron status on FGF23 production and cleavage. Iron deficiency may lead to 1: increased FGF23 transcription, through EPO or HIF1α directly and to 2: increased cleavage of FGF23, mediated by EPO.



In conclusion, increased cFGF23 concentrations, especially in the setting of hypophosphatemia, can be wrongly classified as a FGF23-associated disorder while iFGF23 concentrations may be normal, in the setting of e.g., iron deficiency. Our case illustrates the limitation of cFGF23 assays and we urge clinicians to be aware that cFGF23 concentrations do not necessarily reflect iFGF23 concentrations and that other causes should be considered, including iron deficiency as in our case. Another lesson we learned is that one should be cautious to attribute general complaints like fatigue and

muscle weakness to a mildly decreased serum phosphate concentration, and keep an open mind to alternative diagnoses.

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Figure 1 was created using BioRender.com

Author contributions
Conceptualization: all authors; Formal analysis: AB, DR; Investigation: AB, DR; Resources: BE, MZ; Supervision: BE, MZ; Visualization: AB; Writing – original draft: AB; Writing – review and editing: all authors.

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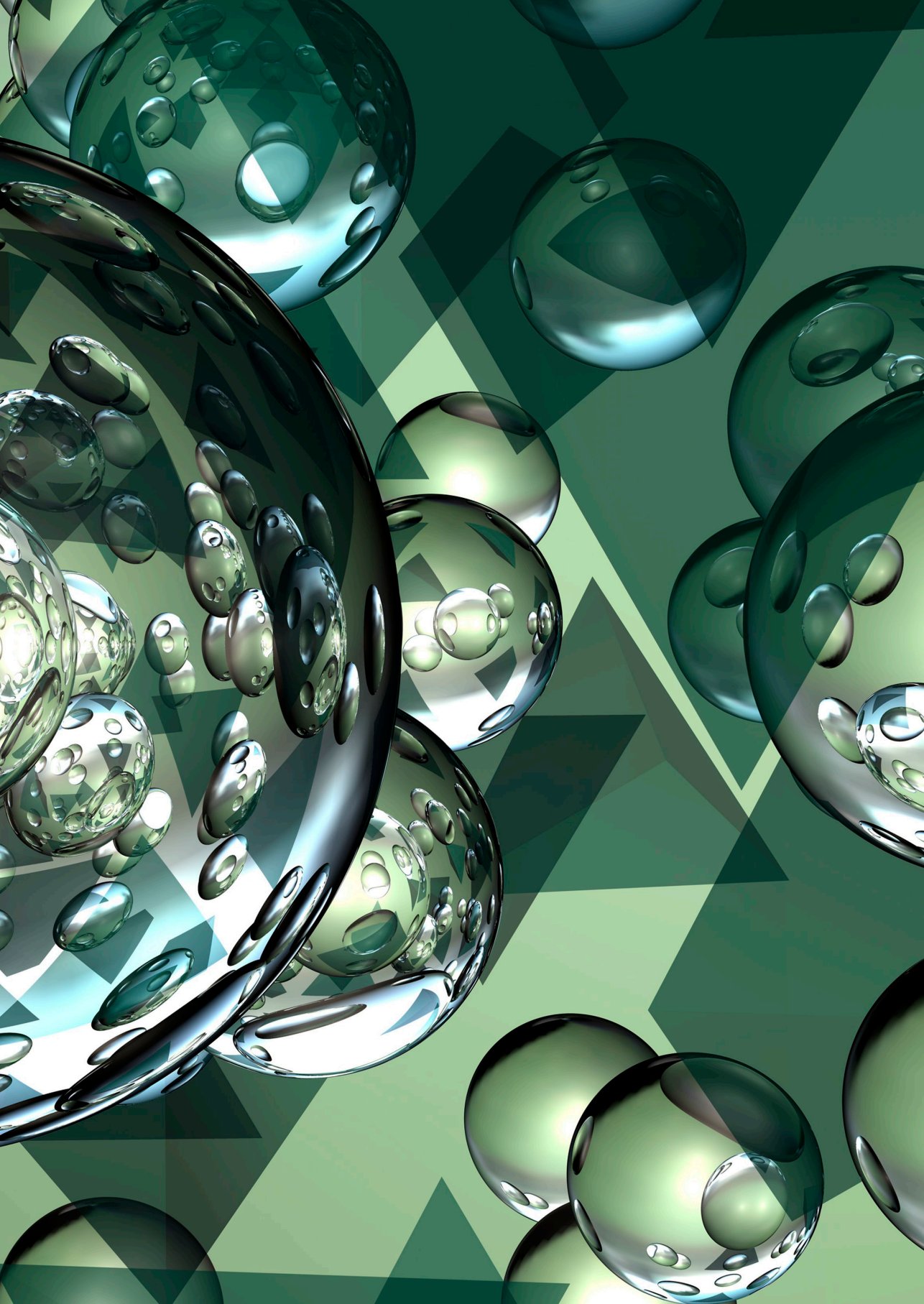
PART IV

Consequences of phosphate disturbances



Disease manifestations and complications in Dutch X-linked hypophosphatemia patients

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ABSTRACT

Context

X-linked hypophosphatemia (XLH) is the most common monogenetic cause of chronic hypophosphatemia, characterized by rickets and osteomalacia. Disease manifestations and treatment of XLH patients in the Netherlands are currently unknown.

Objective

To describe Dutch XLH patients.

Methods

Characteristics of XLH patients participating in the Dutch observational registry for genetic hypophosphatemia and acquired renal phosphate wasting were analyzed.

Results

Eighty XLH patients, including 29 children, were included. Genetic testing, performed in 78.8% of patients, showed a *PHEX* mutation in 96.8% . Median (range) Z-score for height was -2.5 (-5.5; 1.0) in adults and -1.4 (-3.7; 1.0) in children. Many patients were overweight or obese: 64.3% of adults and 37.0% of children. All children received XLH-related medication e.g., active vitamin D, phosphate supplementation or burosumab, while 8 adults used no medication. Lower age at start of XLH-related treatment was associated with higher height at inclusion. Hearing loss was reported in 6.9% of children and 31.4% of adults. Knee deformities were observed in 75.0% of all patients and osteoarthritis in 51.0% of adult patients. Nephrocalcinosis was observed in 62.1% of children and 33.3% of adults. Earlier start of XLH-related treatment was associated with higher risk of nephrocalcinosis and detection at younger age. Hyperparathyroidism longer than six months was reported in 37.9% of children and 35.3% of adults.

Conclusions

This nationwide study confirms the high prevalence of adiposity, hearing loss, bone deformities, osteoarthritis, nephrocalcinosis and hyperparathyroidism in Dutch XLH patients. Early start of XLH-related treatment appears to be beneficial for longitudinal growth but may increase development of nephrocalcinosis.

Keywords

FGF23, phosphate, hypophosphatemia, XLH, X-linked hypophosphatemia

BACKGROUND

X-linked hypophosphatemia (XLH) is the most common genetic cause of chronic hypophosphatemia. It results from loss-of-function genetic variations in the phosphate-regulating endopeptidase homolog X-linked (PHEX) gene, leading to increased circulating fibroblast growth factor 23 (FGF23) levels[1-3]. Excess FGF23 levels can cause hypophosphatemia by reducing renal phosphate reabsorption and decreasing 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$) production[4,5]. Chronic hypophosphatemia can cause multiple problems including muscle weakness and decreased bone mineralisation. XLH is characterized not only by rickets in children and osteomalacia in adults, but also bone pains, increased risk of (pseudo)fractures, dental and hearing problems, osteoarthritis and enthesopathies[6]. Prevalence has been estimated between 1:20,000-60,000 children[7-9]. Conventional treatment consists of active vitamin D and oral phosphate supplementation but is associated with the development of nephrocalcinosis/nephrolithiasis and hyperparathyroidism[6]. The anti-FGF23 antibody burosumab was approved for use in children with XLH in the Netherlands in 2018 and for adults in 2020. The observational registry for genetic hypophosphatemia and acquired renal phosphate wasting in The Netherlands (ORPHOS-NED) has been set up to evaluate retrospectively and prospectively demographic, biochemical, radiological and genetic characteristics, treatment and quality of life of patients with genetic and acquired forms of chronic hypophosphatemia due to renal phosphate wasting. In the current study, we describe for the first time the clinical characteristics of XLH patients in the Netherlands and occurrence of complications including (pseudo)fractures, nephrocalcinosis/nephrolithiasis, and hyperparathyroidism.

METHODS

Population

ORPHOS-NED is an ongoing nationwide observational cohort study on chronic hypophosphatemia, which started inclusion in 2020. Currently, 9 hospitals (academic and non-academic) participate. Adult and pediatric nephrologists and endocrinologists were contacted to identify patients with chronic hypophosphatemia. Patients of all ages with the diagnosis of hypophosphatemic rickets/osteomalacia based on clinical, radiological, biochemical and/or genetic results were included. In addition, patients with chronic hypophosphatemia defined as a serum phosphate level below the lower limit of normal of the reference range in adults and below the age-dependent reference value in patients younger than 18 years of age in two consecutive blood samples taken at least three weeks apart were also included. Patients with chronic hypophosphatemia due to

a known dietary phosphate deficiency or malabsorption, primary hyperparathyroidism or due to a general proximal renal tubulopathy were excluded, as well as patients with hypophosphatemia in acute clinical settings such as refeeding. After informed consent, retrospective data from the first clinical presentation and onwards was collected from medical files. Data in the registry has been – and will be- updated yearly. In the current study, we analyzed the data of adults and children with a diagnosis of XLH who were included in ORPHOS-NED before November 1st 2022. The diagnosis was either confirmed based on the finding of a (likely) pathogenic genetic variation in *PHEX* or suspected based on the phenotype and/or family history. Most pediatric XLH patients in ORPHOS-NED were included at the University Medical Center Groningen, which is the Dutch reference center for burosumab treatment in children.

Patient characteristics

We analyzed the following parameters: clinical characteristics at inclusion (age, sex, family history, height and weight, body mass index (BMI)), genetic testing, medical treatment at time of inclusion, biochemical findings at inclusion (including serum phosphate, calcium, parathyroid hormone (PTH), FGF23, 25-hydroxy-vitamin D (25OHD), 1,25$(OH)_2$D, alkaline phosphatase (ALP), N-terminal propeptide of type I procollagen (P1NP), bone-specific alkaline phosphatase (BALP), C-terminal telopeptide of type I collagen (CTX), ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate (TmP/GFR)) and historical data on renal imaging (nephrocalcinosis, nephrolithiasis), bone deformities, osteoarthritis, orthopedic interventions, fractures, hyperparathyroidism and hearing loss.

To compare height in XLH patients to the general population in the Netherlands, we calculated age- and sex-specific Z-scores[10]. If height and/or weight in adults were not measured in the year of study inclusion, we collected these data reported within 5 years before inclusion. BMI (kg/m^2) was calculated using weight and height. For children, we calculated age- and sex-specific Z-scores for BMI using data from the Fifth National Growth study[11]. In adults, healthy weight, overweight and obesity were defined as a BMI between 18.5-25, 25-30 and greater or equal to 30, respectively. In children, we used age- and sex-specific cut-off values for BMI defined by the Dutch center for youth healthcare[12].

Serum phosphate concentrations are higher in infants than adults, and gradually decrease during childhood and adolescence. To analyze serum phosphate in the total pediatric population, we calculated Z-scores by applying age-related formulas for mean and standard deviation constructed by Verploegen et al.[13]. Hypophosphatemia was defined as Z-score below -2. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation in adults and the Chronic Kidney Disease

in Children Under 25 equation in children[14,15]. TmP/GFR was calculated whenever there was simultaneous measurement of phosphate and creatinine in serum and in a urine portion, irrespective of XLH-related treatment. TmP/GFR was calculated using the formula provided by Barth et al. and the age-related reference ranges for TmP/GFR as calculated by Payne et al. were applied[16,17].

Currently, FGF23 concentrations in the Netherlands are measured by a C-terminal FGF23 (cFGF23) assay (Immutopics, San Clemente, CA, USA) at Amsterdam University Medical Center (upper limit of normal: 125 RU/mL)[18]. This assay measures both intact and C-terminal FGF23[19]. We only analyzed FGF23 concentrations that were measured by this assay. Burosumab treatment can greatly increase FGF23 concentrations[20]. Therefore, patients with FGF23 concentrations measured under burosumab treatment were excluded from analyses on FGF23.

In adults, we compared values for the bone formation markers BALP and P1NP and the bone resorption marker CTX with the age- and sex-specific upper limit of normal. In children, we calculated age- and sex-specific Z-scores. Z-scores above 2 were considered increased.

Hypercalciuria from 24h urinary calcium excretion[21] is defined in the Netherlands as excretion above 7.5 mmol (300 mg) per day. As collection of 24h urine is challenging in children and therefore often not performed, we used the calcium/creatinine ratio in urine samples taken in the year prior to the diagnosis of nephrocalcinosis[21]. We compared these values to age-related 95th percentile reference values as defined by the Dutch Society for Pediatric Nephrology[22].

Reports of hearing loss were collected from the medical records. Data on osteoarthritis was collected from imaging. Data on bone deformities was collected from physical examinations and from imaging. Data on fractures include all fractures ever sustained as reported in the medical history and/or on imaging. Due to the study design, it was not possible to distinguish between fractures and pseudofractures. Data on orthopedic interventions include all orthopedic interventions documented in the medical records. Data on nephrocalcinosis and nephrolithiasis was collected from renal imaging and from medical history.

Statistical analyses

Demographic characteristics are summarized by standard descriptive summaries (e.g., medians and (interquartile) ranges for continuous variables and percentages for categorical variables). The Shapiro-Wilk test was used to assess normality. Associations between categorical variables were assessed with a Chi square test or Fisher's exact

test. Differences in medians between groups were tested using Mann-Whitney U Test. Correlations were analyzed using Spearman's Rank and Kendal's tau b. Correlation analyses were performed between age at start of XLH-related medical treatment and height Z-score at inclusion, age of the first orthopedic intervention, occurrence of nephrocalcinosis and the age it was observed for the first time. In addition, correlations were analyzed between serum phosphate at inclusion and simultaneously measured serum 25(OH)D, $1,25(OH)_2D$, PTH and cFGF23; and between ALP in adults and serum phosphate, 25(OH)D, $1,25(OH)_2D$ and the bone markers P1NP and CTX.

Study parameters were analyzed in all patients. In case of missing data, we report the results from analyses in the study population with complete data.

RESULTS

XLH patient cohort

On November 1st 2022, ORPHOS-NED included 141 patients with chronic hypophosphatemia of whom 80 patients from 7 academic centers were diagnosed with XLH: 29 children and 51 adults. Data on general demographic characteristics of these 80 patients, including sex, height, etc. are shown in **Table 1**. In adults with a BMI measurement available, 32.1% was overweight (BMI between 25-30 kg/m^2), while 32.1% was obese (BMI above 30 kg/m^2). When considering age- and sex-specific cut-off values for BMI in children, 25.9% was overweight and 11.1% obese.

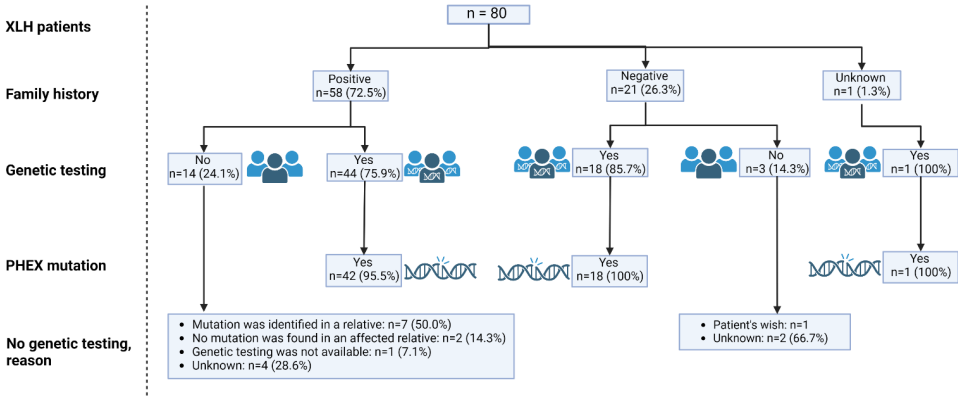
Genetic testing, using Sanger sequencing of *PHEX* or the next generation sequencing panels for hypophosphatemia, was performed in 63 patients (78.8%) and showed in 61 (96.8%) a (likely) pathogenic variation in *PHEX* while in 2 patients no mutation was found (**Figure 1).** The majority of patients (N=58, 72.5%) had a positive family history for hypophosphatemia, bone deformities or short stature. Out of 58 patients with a positive family history, 32 (55.2%) had an affected mother and 9 (15.5%) had an affected father. Maternal grandfathers and grandmothers were affected in 6 (10.3%) patients, while a paternal grandfather was affected in 1 (1.7%) patient and paternal grandmothers were affected in 26 (44.8%) patients.

Table 1. Demographic characteristics of Dutch XLH patients at inclusion in ORPHOS-NED

	Children N=29	Adults N=51
Age, years	9.7 (1.6 to 17.4)	45.8 (19.5 to 76.4)
Female, n(%)	13 (44.8%)	36 (70.6%)
Final height, cm		
Females	-	155 (136 to 177)
Males	-	172 (157 to 184)
Height (Z-score) at inclusion	-1.4 (-3.7 to 1.0) [27]	-2.5 (-5.5 to 1.0) [45]
Males	-1.4 (-3.5 to 0.1) [16]	-1.8 (-3.8 to 0.03) [13]
Females	-1.6 (-3.7 to 1.0) [11]	-2.5 (-5.5 to 1.0) [32]
Body mass index, Z-score	1.2 (-1.4 to 3.5) [27]	-
Males	1.3 (-1.4 to 3.5)	-
Females	0.4 (0.0 to 2.7) [11]	-
Body mass index, kg/m²	-	27.1 (19.5 to 44.0) [28]
Males	-	28.7 (23.6 to 34.0) [8]
Females	-	25.9 (19.5 to 44.0) [20]
Adiposity, n(%)*		
– **Underweight**	1 (3.7%)	-
– **Healthy**	16 (59.3%)	10 (35.7%)
– **Overweight**	7 (25.9%)	9 (32.1%)
– **Obesity**	3 (11.1%)	9 (32.1%)

Continuous values are displayed as median (range), categorical variables are displayed in absolute counts (%). [n] indicates the number of patients in whom these parameters were available. Abbreviation: BMI, body mass index. * In adults, healthy weight, overweight and obesity were defined as a BMI between 18.5-25, 25-30 and ≥30, respectively. In children, age- and sex-specific cut-off values for BMI were applied

Figure 1. Family history and genetic testing in Dutch XLH patients.



Medication use

In adults, 51.0% (n=26) received active vitamin D monotherapy, while 31.4% (n=16) was treated with a combination of active vitamin D and phosphate supplements. One patient received phosphate supplementation without active vitamin D and eight patients were not using XLH-related medication, in the year of inclusion. All children used either active

vitamin D, phosphate supplementation or burosumab in the year of inclusion: 58.6% (n=17) received a combination of active vitamin D and phosphate supplementation, while 79.3% (n=23) used burosumab, indicating that some children were switched from conventional therapy to burosumab during the year of inclusion.

The age at start of XLH-related medical treatment could be traced back in 79.3% of children (n=23) and in 78.4% of adults (n=40). Median age at start was 2.8 years in children (range: 0.3 to 13.3) and 6.9 years in adults (range: 0.0 to 43.3). Only 1 adult patient had never received XLH-related medical treatment. Younger age at start of XLH-related treatment was associated with higher height Z-score at inclusion (Spearman's rho:-0.33, P=0.013).

Laboratory measurements

Table 2 depicts the results from laboratory measurements at the time of inclusion. Hypophosphatemia was present in 93.1% of children and in 86.0% of adults. TmP/GFR was below the age-related lower limit of normal in 92.6% of children. Median (range) C-terminal FGF23 (cFGF23) was 189 (82; 517) RU/mL in children and 109 (58; 1091) RU/mL in adults.

One adult patient had a cFGF23 concentration of 1,091 RU/mL with a serum phosphate concentration of 0.6 mmol/L and an eGFR of 87.9 ml/min/1.73m^2 .

At inclusion, PTH was increased in 24.1% of children and in 20.9% of adults. Among the patients with increased PTH, all children had normal serum calcium concentrations, while 1 adult patient had hypercalcemia and 1 adult patient had hypocalcemia. In addition, 14.3% of all children had a 25(OH)D concentration below 50 nmol/L, compared to 23.3% of adults. Serum 1,25$(OH)_2$D was measured in almost all children with a median (IQR) of 166.5 (100.5) pmol/L. ALP was increased in 44.8% of children and 44.4% of adults.

In children, phosphate Z-score was inversely correlated with serum 25(OH)D concentrations (Spearman's rho: -0.622, *P*=0.018, n=14). Phosphate Z-score was not correlated with serum 1,25$(OH)_2$D, serum cFGF23 or increased PTH. In adults, serum ALP inversely correlated with serum 25(OH)D (Spearman's rho:-0.474, *P*=0.008, n=30), but not with serum 1,25$(OH)_2$D. Serum phosphate inversely correlated with serum C-terminal FGF23 (Spearman's rho:-0.766, *P*=0.027, n=8) but not with serum 25(OH)D, 1,25$(OH)_2$D, PTH or ALP.

In adults, serum ALP positively correlated with serum P1NP (Spearman's rho: 0.731, *P*<0.001, n=26) and with serum CTX concentrations (Spearman's rho: 0.614, *P*=0.001, n=25).

Table 2. Biochemical characteristics of Dutch XLH patients at inclusion in ORPHOS-NED.

	Children N=29	**Adults N=51**
Phosphate, mmol/L	0.86 (0.21)	0.66 (0.19) [50]
Hypophosphatemia, n(%)		43 (86.0%)
Phosphate Z-score	-3.7 (1.6)	
1. **Hypophosphatemia (<-2SD), n(%)**	27 (93.1%)	
Calcium, mmol/L	2.37 (0.13)	2.41 (0.17) [50]
Potassium, mmol/L	4.1 (0.6) [12]	4.1 (0.5) [39]
Sodium, mmol/L	140 (1.8) [12]	140 (2.0) [37]
Magnesium, mmol/L	0.80 (0.07) [23]	0.79 (0.08) [24]
Alkaline phosphatase, U/L	398 (214)	95 (44) [45]
– **Increased, n(%)**	13 (44.8%)	20 (44.4%)
PTH		
– **Normal, n(%)**	15 (51.7%)	32 (74.4%)
– **Increased, n(%)**	7 (24.1%)	9 (20.9%)
– **Unknown, n(%)**	7 (24.1%)	2 (4.7%)
– **Missing, n(%)**	-	8 (15.7%)
25(OH)D, nmol/L	71.5 (33.3) [14]	71.5 (33.3) [30]
– **Deficient, n(%)**	2 (14.3%)	7 (23.3%)
1,25(OH)$_2$D, pmol/L	166.5 (100.5) [24]	79.0 (39.5) [5]
– **Low, n(%)**	1 (4.2%)	-
– **High, n(%)**	8 (33.3%)	-
cFGF23, RU/mL	189 (191) [13)]	109 (97) [7]
– **Increased, n(%)**	11 (84.6%)	3 (42.9%)
Creatinine, µmol/L	38.0 (13.0) [28]	59.0 (17.0) [50]
eGFR, ml/min/1.73m^2 †	123.3 (38.5) [26]	105.4 (27.1) [50]
– **< 60 ml/min/1.73m^2, n(%)**		2 (4.0%)
P1NP, ng/mL	817.5 (222.5) [10]	69.5 (43.3) [26]
– **Increased, n(%)**		13 (50.0%)
P1NP, Z-scores	1.22 (1.44) [8]	-
– **>2SD, n(%)**	2 (25.0%)	
Ctx, µg/L	0.18 (0.14) [10]	0.43 (0.30) [25]
– **Increased, n(%)**		8 (32.0%)
CTx, Z-scores	2.7 (3.4) [8]	-
– **>2SD, n(%)**	5 (62.5%)	
BALP, U/L	189.1 (62.7) [10]	32.0 [1]
BALP, Z-scores	4.4 (3.6) [9]	-
– **> 2SD, n(%)**	8 (88.9%)	
TmP/GFR, mmol/L; median(range)	0.69 (0.22 to 1.48) [27]	0.49 (0.18 to 0.58) [3]
– **< age-related reference range‡, n(%)**	25 (92.3%)	3 (100%)
24h urine calcium, mmol/24h; median(range)	3.0 (2.2 to 3.8) [2]	3.4 (0.6 to 14.5) [34]
Calcium/creatinine ratio, mmol/mmol (urine portion); median(range)	0.83 (0.20 to 6.46) [29]	1.13 (0.96-4.90) [4]

Continuous values are displayed as median (interquartile range) unless otherwise stated, categorical variables are displayed in absolute counts (%). [n] indicates the number of patients in whom these parameters were available. Abbreviation: 25(OH)D, 25-hydroxyvitamin-D; 1,25(OH)$_2$D, 1,25-dihydroxy-vitamin D; BALP, bone-specific alkaline phosphatase; cFGF23, C-terminal fibroblast growth factor 23; CTx, C-terminal telopeptide of type I collagen; eGFR, estimated glomerular filtration rate; P1NP, N-terminal propeptide of type 1 procollagen; PTH, parathyroid hormone; TmP/GFR, ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate. † eGFR was calculated in adults based on the Chronic Kidney Disease Epidemiology Collaboration and in children based on the Chronic Kidney Disease in Children Under 25 equation. ‡the age-related reference ranges for TmP/GFR were derived from Payne et al[17].

Hearing loss, bone deformities and osteoarthritis

Hearing loss was reported in 6.9% of children and in 31.4% of adults. In addition, 11.8% of adults used hearing aids. Results on the age at onset of hearing loss and audiometry are shown in **Table 3**. Data on occurrence of bone deformities and osteoarthritis are shown in **Table 4**. The majority of patients (75.0%), had developed a deformity of the knee (53.8% *genu varum* and 31.3% *genu valgum*). In addition, bowing of femora or of lower legs was present in 20% and 11.3% respectively. Hip deformities were observed in 12.5% of patients and spinal deformities, including scoliosis and increased lumbar lordosis in 7.5% of patients.

Table 3. Hearing loss in children and adults with XLH in the Netherlands

	Total N=80	**Children N=29**	**Adults N=51**
Patient reported hearing loss, n(%)	18 (22.5%)	2 (6.9%)	16 (31.4%)
Hearing loss by audiometry, n(%)	10 (12.5%)	3 (10.3%)	7 (13.7%)
Use of hearing aids, n(%)	6 (7.5%)	-	6 (11.8%)
Age at (reported) hearing loss	35.5 (5.0 to 69.8)	11.5 (8.3 to 14.8)	36.8 (5.0 to 69.8) [15]

Continuous values are displayed as median (range) unless otherwise stated, categorical variables are displayed in absolute counts (%).

Fractures and orthopedic interventions

A fracture history was reported in 22.5% of the total population and in 29.4% of the adult population. Data on age at time of the first (pseudo)fracture and their number and location are shown in **Table 4**. In addition, 43.8% (n=35) of patients had undergone at least one orthopedic intervention, which was performed before the age of 18 years in the majority (68.6%). Data on number and type of orthopedic interventions and age at time of the first orthopedic intervention are shown in **Table 5.** The age at initiation of XLH-related medication did not differ between patients who sustained a (pseudo) fracture or underwent an orthopedic intervention and patients who did not. There was a significant correlation between age at initiation of XLH-related medication and age at the *first* orthopedic intervention (Spearman's rho: 0.413, P=0.029).

Table 4. Bone- and joint characteristics in children and adults with XLH in the Netherlands

	Total N=80	Children N=29	Adults N=51
Bone deformities			
Deformity of the spine, n(%)	6 (7.5%)	1 (3.4%)	5 (9.8%)
Deformity of the hips	10 (12.5%)	2 (6.9%)	8 (15.7%)
Bowing of the femora, n(%)	16 (20.0%)	5 (17.2%)	11 (21.6%)
Deformity of the knee, n(%)	60 (75.0%)	25 (86.2%)	35 (68.6%)
Genu varum	43 (53.8%)	13 (44.8%)	30 (58.8%)
Genu valgum	25 (31.3%)	15 (51.7%)	10 (19.6%)
Bowing of the lower legs, n(%)	9 (11.3%)	5 (17.2%)	4 (7.8%)
Osteoarthritis			
Osteoarthritis of any joint, n(%)	26 (32.5%)	-	26 (51.0%)
Osteoarthritis of the hip, n(%)	15 (18.8%)	-	15 (29.4%)
Osteoarthritis of the knee, n(%)	13 (16.3%)	-	13 (25.5%)
(pseudo)Fractures			
History of a *(pseudo)*fracture, n(%)	18 (22.5%)	3 (10.3%)	15 (29.4%)
Age of first *(pseudo)*fracture	24.0 (5.3 to 54.0)	6.5 (5.3 to 7.0)	34.7 (11.8 to 54.0)
Number of *(pseudo)*fractures	1.5 (1.0 to 14.0)	1.0 (1.0 to 1.0)	2.0 (1.0 to 14.0)
***(pseudo)*Fracture location, n(%)**			
– **Vertebrae**	1 (5.6%)	-	1 (6.7%)
– **Upper arm**	1 (5.6%)	1 (33.3%)	-
– **Hand/wrist**	2 (11.1%)	-	2 (13.3%)
– **Rib**	2 (11.1%)	-	2 (13.3%)
– **Upper leg**	4 (22.2%)	1 (33.3%)	3 (20.0%)
– **Lower leg**	3 (16.7%)	-	3 (20.0%)
– **Foot**	8 (44.4%)	1 (33.3%)	7 (46.7%)

Continuous values are displayed as median (range) , categorical variables are displayed in absolute counts (%).

Table 5. Orthopedic interventions in children and adults with XLH in the Netherlands

	Total N=80	Children N=29	Adults N=51
History of orthopedic intervention, n(%)	35 (43.8%)	10 (34.4%)	25 (42.4%)
Age of first orthopedic intervention	13.5 (1.5 to 58.5)	5.8 (1.5 to 13.5)	16.7 (2.3 to 58.5)
Number of orthopedic interventions	2.0 (1.0 to 10.0)	2.0 (1.0 to 7.0)	3.0 (1.0 to 10.0)
Procedure			
Osteotomy, n(%)	21 (60.0%)	1 (10.0%)	20 (80.0%)
Guided growth, n(%)	7 (20.0%)	5 (50.0%)	2 (8.0%)
Fracture fixation, n(%)	3 (8.6%)	1 (10.0%)	2 (8.0%)
Joint replacement, n(%)	7 (20.0%)	-	7 (28.0%)

Continuous values are displayed as median (range) unless otherwise stated, categorical variables are displayed in absolute counts (%).

Table 6. The prevalence of nephrocalcinosis in children and adults with XLH in the Netherlands

	Total N=80	Children N=29	Adults N=51
Data on renal imaging, n(%)	72 (90.0%)	29 (100.0%)	43 (84.3%)
Nephrocalcinosis			
Nephrocalcinosis, n(%)	35 (47.2%)	18 (62.1%)	17 (33.3%)
Patients' age at diagnosis of nephrocalcinosis, median (range)	9.5 (1.0 to 72.5)	5.5 (1.0 to 15.0)	17.8 (8.0 to 72.5)
Duration of XLH-related medical treatment before discovery of nephrocalcinosis, years, median (range)	4.9 (0.3 to 69.3) [24]	1.7 (0.3 to 14.8) [13]	12.5 (4.0 to 69.3) [11]
Nephrolithiasis			
Nephrolithiasis, n(%)	7 (9.7%)	2 (6.9%)	5 (11.6%)
Patients' age at diagnosis of nephrolithiasis, median (range)	20.7 (11.5 to 55.3)	13.6 (11.5 to 15.8)	51.7 (14.8 to 55.3)

Continuous values are displayed as median (range) unless otherwise stated, categorical variables are displayed in absolute counts (%).

Nephrocalcinosis, nephrolithiasis and hyperparathyroidism

About half of patients with renal imaging data had nephrocalcinosis and 9.7% had nephrolithiasis. Data on patients' age at time of discovery of nephrocalcinosis and duration of XLH-related medical treatment before discovery of nephrocalcinosis are shown in **Table 6.** Of the children with nephrocalcinosis and available urine samples with calcium and creatinine measurements taken the year before or at time of the nephrocalcinosis diagnosis, none had a ratio of calcium-creatinine above the age-related 95th percentile reference value. Three out of the five adults with 24h urine calcium measurements at the time of or in the year before the diagnosis of nephrocalcinosis had hypercalciuria. The age at start of XLH-related medication was inversely correlated with occurrence of nephrocalcinosis (Kendal's tau b:-0.298, P=0.007) and positively correlated with age at diagnosis of nephrocalcinosis (Spearman's rho: 0.468, P=0.011). Results were similar when restricting to patients who started with conventional treatment. Patients who started treatment before the age of 2 years developed nephrocalcinosis more often than patients who started treatment thereafter, 66.7% (10/15) versus 33.3% (14/42) (P=0.035). The time between start of XLH-related medical treatment and discovery of nephrocalcinosis was not significantly different between these two groups (P=0.709). The lifetime prevalence of increased PTH concentrations was 75.9% in the pediatric population and 54.9% in adults (**Table 7**). A period of hyperparathyroidism for more than six months was present in 37.9% (11/29) of the pediatric and in 35.3% (18/51) of the adult population. Concurrent hypercalcemia was observed in 1/11 child and in 7/18 adults. 11.1% (2/18) of adults with prolonged hyperparathyroidism had received calcimimetics, while 22.2% (4/18) of the total group of adults with prolonged hyperparathyroidism and 57.1% (4/7) of adults with hyperparathyroidism with hypercalcemia had undergone total or subtotal parathyroidectomy.

Table 7. The lifetime prevalence of hyperparathyroidism in children and adults with XLH in the Netherlands

	Total N=80	Children N=29	Adults N=51
Occurrence of hyperparathyroidism, n(%)	50 (62.5%)	22 (75.9%)	28 (54.9%)
With normal serum calcium, n(%)	40 (80.0%)	15 (68.2%)	25 (89.3%)
Occurrence of hyperparathyroidism > 6 months, n(%)		11 (37.9%)	18 (35.3%)
Hyperparathyroidism with hypercalcemia, n(%)		1 (9.1%)	7 (38.9%)
Parathyroidectomy, n(%)		-	4 (22.2%)

DISCUSSION

This description of 80 Dutch XLH patients included in ORPHOS-NED provides insight in the manifestations, complications and treatment of this debilitating disease in children and adults in the Netherlands. This study confirms the high prevalence of adiposity, hearing loss, bone deformities, osteoarthritis, nephrocalcinosis and hyperparathyroidism. Early start of XLH-related treatment appears to be beneficial for longitudinal growth but may be harmful for the development of nephrocalcinosis. ORPHOS-NED is an ongoing study but has already been set up in all academic hospitals in The Netherlands and includes a comprehensive review of all paper and electronic medical files of the participants. This approach enables us to provide a detailed description of this patient population and compare standard of care in the Netherlands to international guidelines.

The majority of patients in our study, 72.5%, had a positive family history for hypophosphatemia or a bone related condition, in agreement with a cohort study conducted by Rafaelsen *et al*. who reported a positive family history in 78.6% of pediatric patients with hereditary hypophosphatemia[9]. Still, a considerable number of cases will be sporadic, indicating that XLH should not be excluded based on a negative family history. Decreased growth rate in children is one of the characteristic features of XLH. Beck-Nielsen *et al.* reported a mean Z-score of -1.9 in a cohort of patients with hypophosphatemic rickets, including XLH[23]. Likewise, height at inclusion was decreased in our cohort with a median Z-score of -1.4 in children and -2.5 in adults. We found that age at start of XLH-related medical treatment was inversely correlated with the height Z-score at inclusion. This finding is in agreement with previous studies that showed that early initiation of treatment is beneficial for the growth rate of children[24-26]. This study and previous studies found that adiposity as measured by BMI is prevalent in XLH patients, both in children and in adults[27,28]. Moreover, obesity was more prevalent in this population of children and adults with XLH than in the general Dutch population[29]. Unfavorably, a higher BMI is causally associated with lower serum phosphate in the general population, and negatively effects gait and the lateral trunk lean in XLH (known as waddling gait)[28,30]. BMI may not be a suitable parameter for defining adiposity in XLH

patients with short stature and bone deformities but a recent study in pediatric XLH patients showed increased body fat percentage and decreased muscle mass measured by bioelectrical impedance analysis[31]. Prevention and treatment of obesity should therefore be part of clinical care[6]. It should be noted that there was some missing data of BMI in adults which could cause bias because patients who are overweight may have their weight followed more closely than patients with a BMI in the normal range.

Concerning laboratory measurements, we found that the bone resorption marker CTX and the bone formation marker P1NP were increased in 32.0% and 50.0% of adults respectively, indicating increased bone turnover. This is similar to what was recently reported by Hansen *et al*.[32] In addition, we found that ALP was highly correlated with beta CTX and P1NP in adults. ALP is already used as a marker of osteoblast activity and degree of rickets in XLH[33,34]. Our finding suggests that ALP may be useful in clinical practice as an indicator for increased bone turnover in adult XLH patients. As expected, hypophosphatemia was highly prevalent in our cohort and serum phosphate inversely correlated with cFGF23 in adults. There was no correlation between serum phosphate and cFGF23 in children, possibly because they were on either conventional treatment or burosumab, which may have obscured any existing correlations. In addition, the cFGF23 concentrations at inclusion both in children and adults are much lower than reported in other FGF23-mediated hypophosphatemic disorders such as tumor-induced osteomalacia[35].

Hearing difficulties are one of the medical problems that have been associated with XLH[36]. A study in mice showed that hearing problems may be caused by hypomineralization of the ossicles but other pathways have also been suggested[37,38]. In this study, subjective hearing loss was reported by almost one third of adults and also in 6.9% of children. Data on pure tone audiometry was available for only a few patients. Still, subjective hearing loss was less prevalent than reported by Davies *et al*, who found a prevalence of 48% [39]. Notably, pure tone audiometry detected hearing loss in 76% of subjects in the same study. Taken together, these data show that hearing difficulties are a prevalent, but underreported feature of XLH and that audiometry should be performed routinely in this patient population.

Rickets in childhood can cause bone deformities, that may necessitate orthopedic interventions. Moreover, XLH patients often develop early onset osteoarthritis which may also require orthopedic interventions. Bowing of knees was reported in 75% of the total population, with *genu varum* being more prevalent than *genu valgum*. This is in agreement with recent studies in adult XLH patients by Orlando *et al*. and Mindler *et al*.[28,40]. Osteoarthritis of the hip and knee was reported in about a quarter of adult patients, similar to a cohort study of FGF23-mediated hypophosphatemic rickets by

Beck-Nielsen *et al*. but much lower than reported in other previous studies [23,28,41]. Our retrospective study design is prone to selection bias because extensive radiographic imaging will only have been performed in symptomatic patients. Similarly, Mindler *et al*. studied patients with availability of full length radiographs and Skrinar *et al*. conducted a burden of disease survey among adults with XLH, including questions on presence of osteoarthritis[28,41]. To accurately examine the prevalence of osteoarthritis in XLH, future prospective studies with standardized imaging are essential.

Enthesopathies in XLH, including bone proliferations at ligament attachments or calcification of ligaments are also associated with reduced quality of life[42]. Unfortunately, enthesopathies were not routinely assessed by radiologists in our study population, refraining us from drawing conclusions on their prevalence.

Almost half of our population had undergone one or more orthopedic interventions with osteotomies being the most frequent, in agreement with a previous study by Chesner *et al*. but less than reported in the burden of disease survey by Skrinar *et al*. [41,43]. Moreover, almost 30% of adult XLH patients had sustained at least one fracture, mainly in legs and feet, which is less than reported by Javaid *et al*. who analyzed subjects of a clinical trial and participants in an online survey. In contrast, Beck-Nielsen *et al*. reported a fracture rate of 18% in patients with hypophosphatemic rickets and even a reduced fracture risk compared to controls[1,23]. It should be noted that we did not distinguish between fractures and pseudofractures or Looser's zones.

In a consensus statement, Haffner *et al*. recommend to screen for nephrocalcinosis by performing regular renal ultrasonographies [6]. Nephrocalcinosis is a well-known complication of conventional medical XLH treatment, thought to be the result of medication-induced hypercalciuria. Previous studies also report associations between oral phosphate use and nephrocalcinosis risk but not with use and duration of active vitamin D[44,45]. Ninety percent of our population had undergone renal imaging and nephrocalcinosis was detected in 62% of children and 33% of adults. The prevalence of nephrocalcinosis in our paediatric population is higher than reported recently in a Norwegian cohort of XLH children but overall, the prevalence of nephrocalcinosis ranges between 30 and 70% in the literature[6,9,44]. The median time span of 1.7 years between initiation of medical treatment and discovery of nephrocalcinosis in our pediatric cohort was comparable with the Norwegian cohort[9]. Our finding that one third of adults developed nephrocalcinosis is in agreement with a recent report by Chesher *et al*[43]. Three out of five adult patients with nephrocalcinosis had hypercalciuria the year before or at time of the nephrocalcinosis diagnosis. The absence of hypercalciuria in some children with nephrocalcinosis may be because they were treated with burosumab at that time. Previous studies have questioned the accurateness of the urinary calcium/

creatinine ratio to establish hypercalciuria in children[21]. Interestingly, we found that the age at start of XLH-related medication was inversely correlated with the occurrence of nephrocalcinosis and positively correlated with age at diagnosis of nephrocalcinosis. These findings, together with the lower prevalence in adults and the higher prevalence in children who start treatment before the age of 2 years, suggest that children who start treatment at an early age are more susceptible to develop nephrocalcinosis. This phenomenon has been proposed by others and our findings confirm this relationship[9,46].

Hyperparathyroidism is common in XLH patients and thought to be the result of stimulation of parathyroid cells by FGF23 and by oral phosphate supplementation but could also be related to vitamin D deficiency[6,47,48]. In our cohort, 24% of the pediatric patients and 21% of the adult patients had hyperparathyroidism at inclusion, which is in line with the prevalence reported by Lecoq *et al*[49]. About a third of the total population had developed hyperparathyroidism for a period of more than 6 months. In addition, almost 40% of adults with prolonged hyperparathyroidism developed hypercalcemia, indicating primary or tertiary hyperparathyroidism, and over 20% required parathyroidectomy. Taken together, these results indicate that hyperparathyroidism is prevalent in XLH and reinforces the need for PTH monitoring.

This study has several limitations. ORPHOS-NED identifies potential study participants by approaching pediatric and adult endocrinologists and nephrologists. Consequently, most patients receive medical treatment and are treated in a tertiary center. This study design does not allow us yet to draw conclusions on the prevalence of XLH in the Netherlands or on the XLH phenotype of patients treated in non-academic hospitals. However, inclusion of (non-academic) hospitals is ongoing. In addition, most children in this study were included in the University Medical Center Groningen, which is the reference center for burosumab in children in the Netherlands. For this reason, the phenotype of the children in this study may be more severe than of other pediatric XLH patients in the Netherlands who are not on burosumab treatment. Moreover, so far we collected and analyzed retrospective data, so there was some missing data and fasting status was often unknown. However, by carefully studying both paper and electronic medical records of all included patients in all participating hospitals, we were able to collect most of the data points. Lastly, it is important to note that all laboratory data at time of inclusion were collected irrespective of treatment with burosumab, phosphate and/or active vitamin D supplementation, which will affect TmP/GFR, serum phosphate and $1,25(OH)_2D$ concentrations.

In conclusion, this nationwide cohort of adult and pediatric XLH patients enables us to study manifestations and complications of XLH. Our data show that adiposity, bone deformities, osteoarthritis, hearing loss, nephrocalcinosis and hyperparathyroidism are

highly prevalent in Dutch XLH patients. Fractures occur in a minority of patients, mainly in legs and feet. Moreover, this study confirms that early start of XLH-related treatment is beneficial for longitudinal growth but may pose a risk factor for development of nephrocalcinosis.

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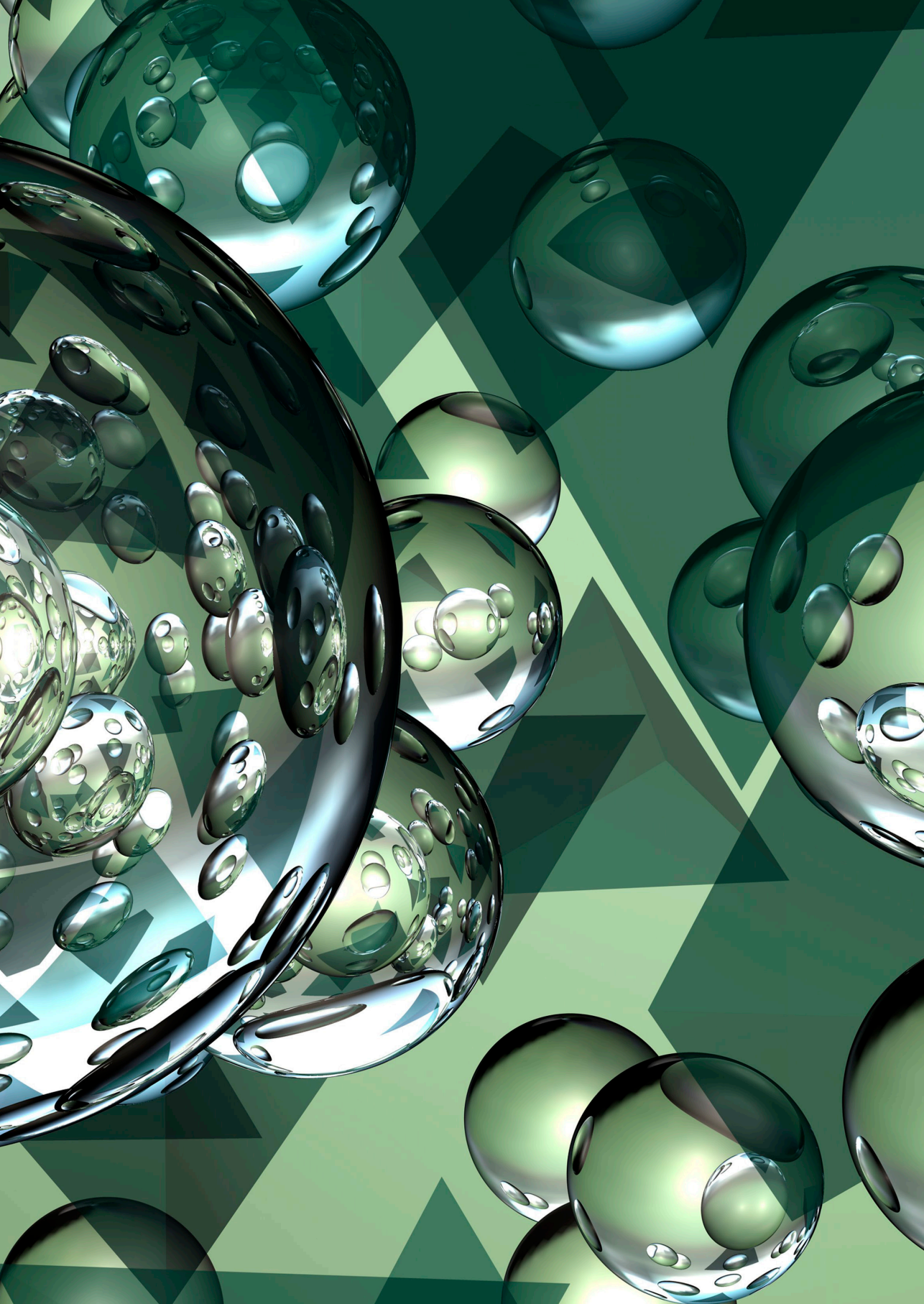

Genetic evidence for a causal role of serum phosphate in coronary artery calcification: The Rotterdam Study

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ABSTRACT

Background

Hyperphosphatemia has been associated with coronary artery calcification (CAC) mostly in chronic kidney disease (CKD), but the association between phosphate levels within the normal phosphate range and CAC is unclear. Our objectives were to evaluate associations between phosphate levels and CAC among men and women from the general population, and assess causality through Mendelian Randomization (MR).

Methods and Results

CAC, measured by electron-beam computed tomography, and serum phosphate levels were assessed in 1889 individuals from the Rotterdam Study. Phenotypic associations were tested through linear models adjusted for age, body mass index, blood pressure, smoking, prevalent cardiovascular disease (CVD) and diabetes mellitus, 25-hydroxyvitamin D, total calcium, C-reactive protein, glucose and total cholesterol:HDL cholesterol ratio. MR was implemented through an allele score including eight phosphate-related single nucleotide polymorphisms. In phenotypic analyses, serum phosphate (per 1-SD) was associated with CAC with evidence for sex interaction (p interaction=0.003) (men β:0.44 (95% CI: 0.30-0.59), p=3x10-9, n=878; women β:0.24 (95% CI: 0.08-0.40), p=0.003, n=1011). Exclusion of hyperphosphatemia, CKD (eGFR<60 mL/min/1.73 m2) and prevalent CVD yielded similar results. In MR analyses, instrumented phosphate was associated with CAC (total population β:0.93 (95% CI: 0.07-1.79), p=0.034, n=1693), even after exclusion of hyperphosphatemia, CKD and prevalent CVD (total population β:1.23 (95% CI: 0.17-2.28), p=0.023, n=1224).

Conclusions

Serum phosphate was associated with CAC in the general population with stronger effects in men. MR findings support a causal relation, also for serum phosphate and CAC in subjects without hyperphosphatemia, CKD and CVD. Further research into underlying mechanisms of this association and sex differences is needed.

Keywords

Phosphate; Coronary artery calcification; Mendelian randomization, chronic kidney disease.

CLINICAL PERSPECTIVE

What is new?

- Higher serum phosphate levels are associated with coronary artery calcifications in the general population with stronger effects in men.
- Mendelian randomization findings support that this association is causal, also for subjects with normal serum phosphate levels and without chronic kidney disease.

What are the clinical implications?

- Serum phosphate and coronary artery calcifications are associated in the absence of chronic kidney disease, hyperphosphatemia and prevalent cardiovascular disease, challenging the concept that only severe hyperphosphatemia, in the setting of chronic kidney disease, is associated with coronary artery calcifications.
- Our findings support a causal role for phosphate in the emerging epidemiological findings that higher serum phosphate levels are associated with increased mortality and cardiovascular events in the general population

INTRODUCTION

Arterial calcification is defined as the deposition of calcium and phosphate in the wall of arteries[1]. It was considered a passive consequence of aging until identified as a risk factor for cardiovascular (CV) events[2]. Current evidence supports that a complex cellular-mediated process underlies arterial calcification[3]. At least, two different layers can calcify: the intima, characteristic of atherosclerosis; and the media, typical of chronic kidney disease (CKD). The arterial mineralization process can result in hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] formation, as is found in bone. Coronary artery calcification (CAC) is one of the most studied calcification processes because of its specificity for atherosclerosis, its correlation to plaque burden[4] and its ability to predict CV events[2,5]. Currently, it is widely accepted that CAC occurs mainly in the intima[6]. Calcification mechanisms are multifactorial, but ectopic bone formation is considered the basis of CAC[7].

Serum phosphate has been related to arterial calcification in several human and animal disorders[8,9], where genetically-induced severe hyperphosphatemia leads to extensive calcification. Hyperphosphatemia-induced calcification was described as the main mechanism of increased mortality in CKD[10]. Similarly, the role of phosphate in CAC has been restricted mainly to hyperphosphatemia[11]. However, Park et al. reported an association of serum phosphate and CAC even within the normal phosphate range in Korean subjects with normal renal function[12]. Interestingly, it has been reported that *increasing yet normal* serum phosphate is also a risk factor for CV morbidity and mortality in the general population[13,14]. These findings have been consistent, although a clear sex difference with stronger associations in men became evident[14,15]. The mechanisms underlying the associations between serum phosphate and CV morbidity and mortality and the reported sex differences remain unexplained.

Two non-mutually exclusive mechanisms have been described for serum phosphate in CAC: a) passive deposition of calcium phosphate, inhibited by pyrophosphate (PPi); b) active induction of osteoblastic differentiation of vascular cells[16]. Additionally, serum phosphate is regulated by several factors including parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_{3)}$ and α-klotho and fibroblast growth factor 23 (FGF23). PTH and $1,25(OH)_2D_3$ exert inductive effects on arterial calcification. α-Klotho and FGF23 exert protective effects on arterial calcification[17,18]. Nevertheless, reports on the association between serum phosphate and CAC in the general population are not consistent as Grønhøj *et al.* did not find an association between serum phosphate and prevalent CAC, but the same group reported an association of serum phosphate with progression of CAC over time[19,20].

As literature on serum phosphate and CAC in the general population is scarce and contradictive[19,21], we aimed to analyse this association in the population-based Rotterdam Study (RS) and test for potential sex differences. Because results from epidemiologic associations can be affected by reverse causation and unmeasured confounding, we also aimed to test for causality applying Mendelian Randomization, a statistical technique whereby genetic variants are used as instrumental variables for the exposure with the purpose of avoiding these sources of bias. If the relevant assumptions are valid, significant MR results can be interpreted as evidence of causality of the exposure on the outcome[22].

METHODS

Study population

The Rotterdam Study (RS) is a prospective cohort study of men and women in the district of Ommoord, Rotterdam, designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described elsewhere [23]. The Rotterdam Study is now composed of four cohorts, named RS-I, RS-II, RS-III and RS-IV (initiated in 1989, 2000, 2006 and 2017, total n~18,000 subjects). Subjects have been assessed at baseline and through follow-up visits. For the current study, we included participants from RS-I (n=7983). Fasting serum phosphate and CAC were assessed during the second follow-up visit of RS-I. A total of 1889 subjects with both measurements available were included in the analyses. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. Data can be obtained upon request. Requests should be directed towards the management team of the Rotterdam Study (datamanagement.ergo@erasmusmc.nl), which has a protocol for approving data requests. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository.

Coronary calcification assessment

Coronary artery calcification was visualized using electron-beam Computed Tomography (EBT; C-150 Imatron Scanner, GE Healthcase, South San Francisco, CA). From the level of the aortic root through the heart, 38 images were obtained with a 100-ms scan time and a

3-mm slice thickness. During one breath hold, images were acquired at 80% of the cardiac cycle by using echocardiographic triggering. Quantification of coronary calcification was performed with AccuImage software (AccuImage Diagnostics Corporation, South San Francisco, CA). The presence of calcification was defined as a *minimum* of 2 adjacent pixels (area=0.65 mm^2) with a density > 130 HU. Following Agatston's method, calcium scores were calculated by multiplying the area in mm^2 of individual calcified lesions with a factor based on the peak density of the lesion[24]. The total score for the entire epicardial coronary vascular system comprised the sum of the scores for all individual lesions. Scores were log transformed (+1) to reduce the sensitivity to observations with extremely high CAC values.

Laboratory measurements

Fasting blood samples were obtained in the second follow-up visit and serum phosphate was measured with a method based on the formation of ammonium phosphomolybdate, that corresponds to the inorganic fraction of total phosphorus. Total calcium concentrations were assessed through a colorimetric o-cresolphthalein complex one method (Merck Diagnostica, Amsterdam, The Netherlands). Levels of 25-hydroxyvitamin D (25(OH) D) were determined through an electrochemiluminescense immunoassay, adjusting for seasonality through cosinor models. Creatinine concentrations were determined through a sarcosine-based colorimetric assay and standardized. Subsequently, the Chronic Kidney Disease Epidemiology Collaboration equations were applied to estimate glomerular filtration rate (eGFR). C-reactive protein (CRP), glucose, cholesterol and alkaline phosphatase (ALP) levels were measured through standard methods[25,26]. Ionized calcium was measured through a colorimetric detection assay using Hitachi 917 (Roche, Mannheim, Germany). Assessments for ALP and ionized calcium were done at baseline visit and are therefore not simultaneous with serum phosphate.

Genotyping

Participants were genotyped in the Illumina HumanHap550 BeadChip SNP array. Variants were filtered on call rate <98%, minor allele frequency<0.01 and Hardy Weinberg equilibrium (HWE) $P<10^{-6}$, and imputed to the Haplotype Reference consortium panel, release 1.1. Genetic instruments for serum phosphate were selected from genome-wide association analysis (GWAS) significant independent single nucleotide polymorphisms (SNPs) identified in the European GWAS by Kestenbaum et al. and in a Japanese GWAS by Kanai et al.[27,28]. Variants selected for analyses were checked for HWE p>0.05 for genotyped SNPs, imputation quality for imputed SNPs (R^2>0.8) and allele frequencies for palindromic SNPs to decrease the possibility of strand inconsistencies.

Other covariates

Prevalent cardiovascular disease was defined as prevalent myocardial infarction,

revascularization, stroke and heart failure. Prevalent cardiovascular disease was assessed using general practitioner's records and hospital discharge letters[29]. Smoking status was assessed during home interviews. Blood pressure, height and weight were measured during visits. BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Prevalent diabetes mellitus was assessed using general practitioners' records, information on antidiabetic medication use and fasting blood glucose levels[30].

Statistical analysis

Phenotypic associations

The association between serum phosphate and CAC as a continuous variable was assessed through linear regression models. The analysis was stratified according to eGFR (< 60 mL/min/1.73 m^2: chronic kidney disease (CKD)). Serum phosphate was assessed continuously and in quintiles. In addition, we tested the linearity of the association between serum phosphate and CAC using restricted cubic spline (RCS) functions with 5 default knots (5th, 27.5th, 50th, 72.5th, and 95th percentile)[31,32]. We tested if the calcium*phosphate product (Ca*P) was associated with CAC, as previously reported in CKD[33]. In addition, we assessed the association between serum phosphate and CAC as a categorical variable through prevalence ratios (PR), for CAC scores greater than 100, 300, 400 and 1000 [34]. We included interaction terms of phosphate with sex in age-adjusted models to explore potential sex differences in the association between phosphate and CAC. When a sex-difference in serum phosphate and CAC was confirmed, we performed sex-stratified analysis. Because calcium is synergistic to phosphate in arterial calcification[33], we assessed the relation between serum phosphate and ionized calcium.

Model I included adjustments for age, BMI and smoking. Model II included also blood pressure, 25(OH)D, total serum calcium, CRP, total cholesterol:HDL cholesterol ratio and glucose levels, prevalent CVD and prevalent diabetes mellitus.

Sensitivity analyses

We restricted the analyses to subjects without hyperphosphatemia (hyperphosphatemia: serum phosphate>1.45 mmol/L, >4.5 mg/dL), without CKD and without prevalent CVD. To explore for interaction, we created 25(OH)D categories splitting at 48 nmol/L which is a threshold related to CVD in the Dutch population[35]. We assessed whether serum tissue non-specific ALP was associated with CAC. ALP generates phosphate through hydrolysis of inorganic PPi, a potent arterial calcification inhibitor, and has been independently related to CAC in the general population, in CVD and in CKD[36].

Mendelian randomization

To test for causality, we assessed whether phosphate was associated with continuous CAC[22]. We selected eight SNPs, assumed an additive model and built a phosphate-

increasing allele score aligning the alleles: a higher score predicted a higher serum phosphate[37]. Scores (as a single instrument) are associated with lower risk of weak instrument bias than the simultaneous use of multiple SNPs[37]. The SNPs included in the score were derived from a GWAS meta-analysis within CHARGE Consortium composed of individuals of European ancestry, and a recent GWAS within the Biobank Japan Project (BBJ) composed of individuals of East Asian ancestry[28,38]. In the former, the population under study was part of the discovery sample of the GWAS, which could result in bias from winner's curse. However, all SNPs reported in the above mentioned GWA studies have been replicated in a UK biobank GWAS. Summary statistics of this GWAS are publicly available[39].

To properly account for uncertainty in imputed SNPs, genotypes were extracted from dosage files and therefore its values span between 0-2, reflecting the probability of getting up to 2 risk (phosphate-increasing) alleles. If the SNP was genotyped, we report HWE test; otherwise the imputation quality (R^2) is displayed.

Palindromic SNPs were checked for allele frequency concordance between RS and the GWAS catalogue. In addition, one SNP mapping to the non pseudoautosomal region of the X-chromosome was coded as 0/2 in men (for 0/1 risk allele) and as 0/1/2 in women, following recent guidelines when assuming a pattern of X-chromosome inactivation[40]. Inclusion of correlated SNPs, in linkage disequilibrium, is a potential source for bias in standard MR analyses without covariance matrix, leading to increased type I error rates. Therefore, we included only independent SNPs in the score, applying a threshold of r^2 <0.01.

We used the score to genetically predict serum phosphate and tested for causality applying a two stage least square regression (*tsls)*, where first stage regresses the exposure on the instruments and second stage regresses the outcome on the fitted values of the exposure estimated in first stage[37]. We applied robust standard errors. We assessed MR assumptions as follows:

> Assumption N°1: the instrument must be associated with the exposure, *relevance* condition. We regressed serum phosphate levels on phosphate SNPs/score in the population with serum phosphate levels available and with serum phosphate and CAC levels available. We considered *β*, *p* and F-statistic. In general, an instrument with an F-statistic less than 10 is considered a weak instrument and a higher F-statistic reflects a better instrument. However, no cut-offs should be made as bias is a continuous phenomenon[22,37].

Assumption N°2: the instrument must not be associated with potential confounders, *independence* condition. We regressed potential confounders on phosphate scores. Evidently, it is not possible to assess the association of instruments with unmeasured confounders.

Assumption N°3: the instrument must be related to the outcome only through the exposure, meaning the absence of horizontal pleiotropy, *exclusion-restriction* condition. To test this assumption, both a frequentist and a Bayesian approach were applied, the latter as sensitivity analysis. MR-Egger regression was not applied due to our one-sample setting and its low statistical power when the SNP-exposure associations are homogenous[41]. We implemented instead an adaptive *lasso* regression (*sivreg*, Stata) that provides estimates while allowing less than 50% of instruments to be invalid by horizontal pleiotropy[42].

Analyses were performed with unweighted scores. Although weighted scores may increase power, use of internal weights derived from the data should be avoided due to the severe bias that this approach induces and the Rotterdam Study represented ~40% of the CHARGE GWAS[37,38].

To test if results from the score were driven by only one SNP, we applied the leave-one-out approach, excluding one SNP at a time from the score and testing for each reduced score whether genetically predicted phosphate was still associated with CAC. This penalization technique is considered a robust method[43]: if results are driven by only one SNP a high index of pleiotropy should be suspected and properly assessed. To obtain results for the Sargan test, which is an indirect measure of heterogeneity among instruments, we did not apply robust standard errors to the leave-one-out analysis. Similar to phenotypic associations, we performed subgroup analyses excluding participants with hyperphosphatemia, CKD and prevalent CVD.

Sensitivity analysis

In addition of applying a single score, we genetically predicted phosphate through the combination of all SNPs simultaneously (joint instruments analyses). This approach might have more power but might suffer from weak instrument bias[37]. We applied in this setting the Sargan test, an overidentification test, to assess whether all instruments included in the regression are valid in linear combination[44] and not correlated with error terms. Additionally, Sargans' provides a test of heterogeneity among instruments[43]. We also applied the leave-one-out approach to the joint instruments analysis.

Consistent with ancestry of participants, we built a score derived only from SNPs from CHARGE meta-analysis (EUR-score), and tested for reproducibility of results.

Finally, we applied a Bayesian approach designed for one-sample setting that allows several SNPs to exert pleiotropic effects and that does not rely in the assumption of no correlation between SNP strength and pleiotropic effects. The method is described in Supplementary information for Bayesian approach to pleiotropy assessment. This approach allows the implementation of pleiotropic effects for a subset (49%) of SNPs, incorporated in their prior distribution, and applies variational Bayes through a modified Markov Chain Monte Carlo to estimate the posterior mean and its 95% Credible Interval[45]. Genotypes were centered to improve convergence. Analyses were performed after imputing missing values through multiple imputation with chained equations. We used SPSS (version 21.0, Armonk, NY: IBM Corp), Stata (version 16, College Station TX: Stata Corp LP) and R (version 3.5.0; Vienna, Austria). A two-sided $p<0.05$ was considered significant.

RESULTS

The general characteristics of the study population are shown in Table 1. Mean serum phosphate levels were higher in women than in men (P_{women}: 1.18 mmol/L (3.65 mg/dL); P_{men}: 1.02 mmol/L (3.16 mg/dL); $p_{t\text{-}test} < 0.001$ (data not shown)) (reference range of serum phosphate: 0.8-1.4 mmol/L= 2.5-4.5mg/dL). Median CAC score levels were higher in men than in women (CAC_{men}: 310.2; CAC_{women}: 54.7: $p_{M\ Whitney} < 0.001$). Total calcium and calcium*phosphate product was positively associated with serum phosphate in both sexes while ionized calcium was negatively associated with serum phosphate in women only. All results are expressed per 1-SD increase of serum phosphate (0.16 mmol/l=0.49 mg/dL), unless otherwise stated.

Table 1. General characteristics of study population, per quintiles of fasting phosphate levels.

	Men					**Women**				
	Phosphate in quintiles					Phosphate in quintiles				
	1	2	3	4	5	1	2	3	4	5
N	175	176	175	176	176	202	202	202	202	203
(Phosphate in mmol/L)	(0.83)	(0.95)	(1.02)	(1.09)	(1.21)	(0.98)	(1.11)	(1.17)	(1.24)	(1.37)
Age (years)	70.8	71.3	70.6	70.6	70.7	70.7	70.2	71.2	70.8	70.6
BMI (kg/m^2)	26.8	26.7	26.3	26.8	26.1	29.1	27.6	27.3	26.9	26.3
Ever smoke (%)	93.1	89.1	93.1	94.3	94.3	48.8	52.0	55.9	58.2	53.0
Systolic BP (mm Hg)	145.1	143.4	148.0	146.1	144.0	145.8	143.9	147.9	139.3	142.5
Diastolic BP (mm Hg)	76.9	76.5	82.5	78.1	76.5	78.4	75.8	79.3	73.9	74.4
Ionized Ca^{++} (mmol/L)*	1.29	1.29	1.29	1.29	1.28	1.31	1.30	1.29	1.30	1.28
Calcium (mmol/L)	2.39	2.41	2.40	2.40	2.42	2.43	2.43	2.44	2.44	2.46
Ca*P product(mmol^2/L^2)	1.98	2.28	2.44	2.61	2.94	2.38	2.69	2.87	3.04	3.38
ALP (U/L)*	79.6	76.5	75.6	74.4	75.0	80.5	76.7	77.7	80.3	76.5
25(OH)D (nmol/L)	66.7	63.1	65.6	60.8	60.5	49.6	51.5	48.4	49.2	50.8
CAC score	446.7	668.5	696.9	840.3	1059.8	234.2	193.4	319.0	342.6	279.0
CRP (mg/L)	3.95	3.37	3.74	3.64	5.20	4.78	3.77	3.57	3.36	3.05
Glucose (mmol/L)	6.08	6.00	6.14	6.09	6.02	6.12	5.77	5.80	5.77	5.64
eGFR (mL/min/1.73 m^2)	72.7	72.5	73.9	73.1	74.7	71.8	72.6	71.0	71.8	72.8
Chol to HDL ratio	4.78	4.86	4.82	4.65	4.45	4.39	4.40	4.36	4.21	4.10
Prevalent CVD (%)	12.0	17.6	16.6	17.0	19.9	6.0	7.4	4.5	8.4	6.4
Prevalent DM (%)	13.1	13.6	13.7	17.0	15.9	16.8	11.9	12.4	10.4	9.9

Continuous values are displayed as means; categorical variables are displayed in percentages.* Ionized calcium and alkaline phosphatase (ALP) levels were not assessed simultaneous with serum phosphate levels. BMI, body mass index; BP, blood pressure; ionized Ca++, ionized calcium levels; calcium, total calcium levels; Ca*P product, total calcium x phosphate levels; ALP, alkaline phosphatase levels; 25(OH)D, 25-hydroxyvitamin D levels; CRP, C-reactive protein; prevalent DM, prevalent diabetes mellitus; eGFR, estimated glomerular filtration rate; Chol to HDL ratio, total cholesterol to HDL cholesterol ratio; prevalent CVD, prevalent cardiovascular disease; prevalent DM, prevalent diabetes mellitus.

Results from phenotypic associations

CAC as continuous trait

Table 2 shows that serum phosphate was associated with CAC in the total population after adjustments for Model I (*β*: 0.37, 95% CI: 0.26-0.48, p=3x10^{-11}, n=1889). We found a significant interaction between serum phosphate and CAC across sexes ($p_{interaction}$=0.003). Sex-stratified analyses showed that the association between serum phosphate and CAC was stronger in men (*β*: 0.52, 95 CI: 0.38-0.67, p=5 x10^{-12}, n=878) than women (*β*:0.22, 95 CI: 0.06-0.38, p=0.006; n=1011). Further adjustments (Model II) induced a slight attenuation of the association in men (men *β*: 0.44, 95 CI: 0.30-0.59, p=3x10^{-9}; women *β:* 0.24, 95 CI: 0.08-0.40, p=0.003).

Table 2. Association between serum phosphate levels and coronary artery calcification scores

	Model I			**Model II**		
	n	*β* (95% CI)*	*P-value*	n	*β* (95% CI)*	*P-value*
Men	878	0.52 (0.38 to 0.67)	<0.001	878	0.44 (0.30 to 0.59)	<0.001
Women	1011	0.22 (0.06 to 0.38)	0.006	1011	0.24 (0.08 to 0.40)	0.003
Total	1889	0.37 (0.26 to 0.48)	<0.001	1889	0.34 (0.23 to 0.45)	<0.001

* *β*s were obtained from linear regression models and expressed per 1-SD increase in phosphate (0.16 mmol/l=0.49 mg/dL). Model I: adjusted for age, BMI, smoking. Model II: adjusted for age, BMI, blood pressure, smoking, prevalent cardiovascular disease, prevalent diabetes mellitus and serum levels of 25-hydroxyvitamin D, total calcium, C-reactive protein, total cholesterol to HDL cholesterol ratio and glucose.

The stratified analyses (**Table 3**) showed that serum phosphate was associated with CAC across the spectrum of kidney function in men (eGFR≥ 60 mL/min/1.73 m^2 *β*: 0.53, 95 CI: 0.35-0.70, p<0.001, n=736; eGFR<60 mL/min/1.73 m^2 *β*: 0.53, 95 CI: 0.31-0.75, p<0.001, n=142); while in women this association was constrained to normal eGFR (eGFR≥ 60 mL/min/1.73 m^2 *β*: 0.22, 95 CI: 0.04-0.39, p=0.016, n=839; eGFR<60 mL/min/1.73 m^2 *β*: 0.25, 95 CI: -0.17-0.66, p=0.238, n=172). Adjustments for Model II induced a slight attenuation in men (eGFR ≥ 60 mL/min/1.73 m^2 *β*: 0.44, 95 CI: 0.27-0.62, p<0.001; eGFR<60 mL/min/1.73 m^2 *β*: 0.45, 95 CI: 0.21-0.68, p=0.001).

The analyses in quintiles suggested a threshold for the association of serum phosphate and CAC (**Table 4**): setting the first quintile as reference, men with serum phosphate above 1.09 mmol/L displayed a significant trend for higher CAC (*β* for fourth quartile: 0.87, 95 CI: 0.46-1.28, p<0.001; *β* for fifth quintile: 1.18, 95 CI: 0.77-1.59, p<0.001; p_{trend} =3x10^{-10}, n=878). For women the threshold was above 1.37 mmol/L (*β* for fifth quintile 0.67, 95 CI: 0.22-1.11, p=0.003; p_{trend} =0.002, n=1011). A 5 knot RCS function did not find evidence for a non-linear association between serum phosphate and CAC in the total

population and in men and women separately (**Supplementary Figure 1**).

Table 3. Association between serum phosphate levels and coronary artery calcification scores, stratified by eGFR

	eGFR≥60 mL/min/1.73 m²*			eGFR<60 mL/min/1.73 m²*		
	n	*β* (95% CI) †	*P-value*	n	*β* (95% CI) †	*P-value*
Model I						
Men	736	0.53 (0.35 to 0.70)	<0.001	142	0.53 (0.31 to 0.75)	<0.001
Women	839	0.22 (0.04 to 0.39)	0.016	172	0.25 (-0.17 to 0.66)	0.238
Total	1575	0.36 (0.24 to 0.49)	<0.001	314	0.42 (0.20 to 0.64)	<0.001
Model II						
Men	736	0.44 (0.27 to 0.62)	<0.001	142	0.45 (0.21 to 0.68)	<0.001
Women	839	0.22 (0.05 to 0.40)	0.011	172	0.30 (-0.12 to 0.72)	0.154
Total	1575	0.33 (0.21 to 0.46)	<0.001	314	0.36 (0.14 to 0.58)	0.002

* eGFR estimated from creatinine-based Chronic Kidney Disease Epidemiology Collaboration equations. † *β*s were obtained from linear regression models and expressed per 1-SD increase in phosphate (0.16 mmol/l=0.49 mg/dL). Model I: adjusted for age, BMI, smoking. Model II: adjusted for age, BMI, blood pressure, smoking, prevalent cardiovascular disease, prevalent diabetes mellitus and serum levels of 25-hydroxyvitamin D, total calcium, C-reactive protein, total cholesterol to HDL cholesterol ratio and glucose.

Table 4. Association between serum phosphate levels and coronary artery calcification scores, per quintiles of phosphate levels

Men					**Women**		
n	Phosphate levels mean (range)*	*β* (95% CI) †	*P-value*	n	Phosphate levels mean (range)*	*β* (95% CI)†	*P-value*
175	0.83 (0.63-0.91)	1 (Ref)		202	0.98 (0.74-1.06)	1 (Ref)	
176	0.95 (0.91-0.98)	0.28 (-0.13 to 0.69)	0.178	202	1.11 (1.06-1.14)	0.04 (-0.40 to 0.47)	0.869
175	1.02 (0.98-1.05)	0.37 (-0.04 to 0.78)	0.078	202	1.17 (1.14-1.20)	0.03 (-0.41 to 0.47)	0.889
176	1.09 (1.05-1.13)	0.87 (0.46 to 1.28)	<0.001	202	1.24 (1.20-1.28)	0.26 (-0.18 to 0.69)	0.247
176	1.21 (1.13-2.47)	1.18 (0.77 to 1.59)	<0.001	203	1.37 (1.28-1.70)	0.67 (0.22 to 1.11)	0.003
p trend		<< 0.001				0.002	

* Phosphate quintiles are expressed in mmol/L. † Betas were obtained from linear regression models. First quintile of phosphate was set as reference. Analyses were adjusted for age, BMI and smoking.

Table 5. Association between serum calcium*phosphate product levels and coronary artery calcification scores, per quintiles of calcium*phosphate product levels

Men					**Women**		
N	Product mean (range)*	*β* (95% CI)†	*P-value*	n	Product mean (range)*	*β* (95% CI)†	*P-value*
175	1.97 (1.50-2.16)	1 (Ref)		202	2.35 (1.67-2.57)	1 (Ref)	
176	2.27 (2.16-2.36)	0.03 (-0.37 to 0.44)	0.868	202	2.68 (2.58-2.77)	0.001 (-0.43 to 0.44)	0.995
175	2.44 (2.36-2.52)	0.49 (0.09 to 0.90)	0.017	202	2.86 (2.77-2.96)	0.29 (-0.14 to 0.73)	0.185
176	2.62 (2.52-2.71)	0.77 (0.37 to 1.18)	<0.001	202	3.05 (2.96-3.15)	0.47 (0.04 to 0.91)	0.034
176	2.96 (2.71-6.57)	1.17 (0.77 to 1.58)	<0.001	203	3.40 (3.16-4.20)	0.64 (0.19 to 1.08)	0.005
p_{trend}		<< 0.001				0.001	

* Calcium*phosphate product levels are expressed in $mmol^2/L^2$. † Betas were obtained from linear regression models. First quintile of calcium*phosphate product level was set as reference. Analyses were adjusted for age, BMI and smoking.

Similarly, the results of the calcium*phosphate product with CAC (**Table 5**) suggested threshold values: setting the first quintile as reference, men with a product above 2.44 $mmol^2/L^2$ displayed a significant trend for higher CAC (*β* for third quintile: 0.49, 95 CI: 0.09-0.90, *p*=0.017, *β* for fourth quintile: 0.77, 95 CI: 0.37-1.18, *p*<0.001, *β* for fifth quintile: 1.17, 95 CI: 0.77-1.58, *p*<0.001; $p_{trend} = 5x10^{-11}$, n=878). In women the threshold was above 3.05 $mmol^2/L^2$ (*β* for fourth quintile: 0.47, 95 CI: 0.04-0.91, *p*=0.034, *β* for fifth quintile 0.64, 95 CI: 0.19-1.08, *p*=0.005; p_{trend} =0.001, n=1011). All coefficients are per unit increase in calcium*phosphate product.

ALP was not associated with CAC (men *β:* -0.001, 95 CI:-0.009 to 0.007, *p*=0.873, n=574; women *β*:-0.005, 95 CI:-0.012 to 0.001, *p*=0.094, n=747) (data not shown).

CAC as a categorical trait

After adjustments for Model II (**Supplementary Table 1**), each 1-SD increase in serum phosphate (0.16 mmol/L) was associated with an increased PR for CAC>100 of 8% in men only ((95 CI: 4%-12%), $p=6x10^{-5}$); a PR for CAC>300 of 10% in men only (95 CI: 5%-15%, $p=2x10^{-4}$); a PR for CAC>400 of 10% in men only (95 CI: 5%-16%, $p=2x10^{-4}$), and a PR for CAC>1000 of 20% in men (95 CI: 12%-28%, $p=4x10^{-7}$) and 36% in women (95 CI: 18%-55%, *p*<0.001).

Sensitivity analyses

After exclusion of subjects with hyperphosphatemia, serum phosphate was associated with CAC in both men (*β*: 0.53, 95 CI: 0.37-0.69, $p=1x10^{-10}$, n=873) and women (*β*: 0.21, 95 CI: 0.03-0.40, *p*=0.020, n=974) (Supplementary Table 2). In men, results from Model II

showed a slight attenuation (men: *β*:0.46, 95 CI: 0.31-0.62, $p=1x10^{-8}$). Moreover, exclusion of CKD and hyperphosphatemia did not change the association between phosphate and CAC (men *β*: 0.53, 95 CI: 0.35-0.71, $p=1x10^{-8}$, n=733; women *β*: 0.22, 95 CI: 0.03-0.42, *p*=0.026, n=808). These results were slightly attenuated in men after adjustments for Model II (men *β*: 0.45, 95 CI: 0.27-0.63, $p=1x10^{-6}$). Lastly, after exclusion of CKD, hyperphosphatemia and prevalent CVD, the association between serum phosphate and CAC remained (men: *β*: 0.55, 95 CI: 0.35-0.74, $p=4x10^{-8}$, n=627; women: *β*: 0.20, 95 CI: 0.0003-0.40, *p*=0.050, n=765). Adjustments for Model II yielded similar results (men: *β*: 0.50, 95 CI: 0.31-0.69, $p=5x10^{-7}$; women: *β*: 0.22, 95 CI: 0.02-0.43, *p*=0.029). We found no evidence of effect modification by 25(OH)D (data not shown).

Results from Mendelian Randomization analyses

No evidence of departure from HWE was observed in genotyped SNPs; for imputed SNPs, the quality (R^2) was above 96%. No frequency/strand inconsistency between the original GWAS and RS was detected for the only palindromic SNP (rs2970818; A/T); therefore, it was included in the score (**Supplementary Table 3**)[27].

Concerning MR first assumption, the strength of the instruments was tested regressing serum phosphate levels (expressed in *entire units of mmol/L*) on the SNPs in all subjects with serum phosphate levels available and further restricted to those with serum phosphate and CAC (**Supplementary Table 4**). The allele score was significantly associated with serum phosphate in both the whole cohort with available phosphate levels and in the subset of the population with available phosphate and CAC levels. The F statistic for the allele score was above 25, meaning results from MR analyses should not be affected by weak instrument bias[37]. Three SNPs from BBJ showed a significant association with serum phosphate in the whole cohort but further restriction to subjects with serum phosphate & CAC decreased power and the associations were no longer significant. Therefore, we first built a score including all eight SNPs and subsequently applied sensitivity analyses.

Concerning MR second assumption, we regressed potential confounders on the phosphate scores: the eight-SNP score and the five-SNP EUR-score (**Supplementary Table 5**). We found no association of the scores with total calcium, 25(OH)D, CRP, total cholesterol:HDL cholesterol ratio and glucose; nor with BMI, smoking, prevalent CVD or DM.

Allele score analyses

Figure 1 shows the results of the *tsls* regression of phosphate, genetically predicted through the unweighted score, and CAC adjusted for age, sex and 10 principal components in the whole cohort. The allele score has been scaled to be associated to 1-SD of serum phosphate (0.16 mmol/l=0.49 mg/dL). A significant relation was found

between the unweighted phosphate score and CAC (*β*: 0.93, 95 CI: 0.05-1.82, *p*=0.039). Sex-stratified analysis suggested that the association between genetically predicted phosphate and CAC was more consistent in men than in women (men *β*: 1.31, 95 CI: -0.02-2.64, p=0.053, n=782 ; women *β*: 0.56, 95 CI:-0.61-1.75, p=0.347, n=911).

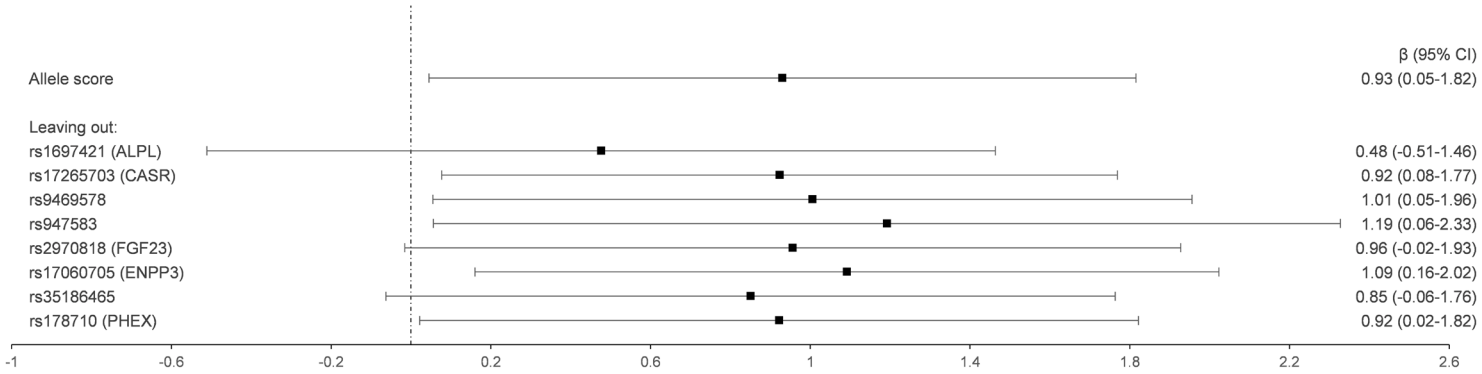
When we applied the leave-one-SNP-out approach (from the score), we found that results lost significance after extracting rs1697421 (*β*: 0.48, 95 CI:-0.51-1.46, p=0.344); rs2970818 (*β*: 0.96, 95 CI:-0.02-1.93, p=0.054) and rs35186465 (*β*: 0.85, 95 CI:-0.06-1.77, p=0.068), one at-a-time.

Subgroup analysis according to serum phosphate levels, kidney function and CVD
The unweighted phosphate score remained associated to CAC after exclusion of hyperphosphatemia (*β*: 1.10, 95 CI: 0.10-2.10, p=0.031, n=1659), after exclusion of hyperphosphatemia and CKD (*β*:1.30, 95 CI: 0.33-2.27, p=0.009, n=1377) and after exclusion of hyperphosphatemia, CKD and prevalent CVD (*β*: 1.23, 95 CI: 0.17-2.28, p=0.023, n=1244) **(Figure 2**, **Supplementary Table 6**).

Sensitivity analyses
Joint instruments analyses and Sargan statistics
Phosphate genetically predicted through all eight SNPs simultaneously (**Supplementary Table 7**) was associated with CAC (*β*:0.81, 95 CI: 0.04-1.58, *p*=0.038). The exclusion of one SNP at-a-time yielded similar results as their exclusion from the score: phosphate is not associated with CAC if the following SNPs are excluded, one at a time: rs1697421, rs2970818 and rs35186465.

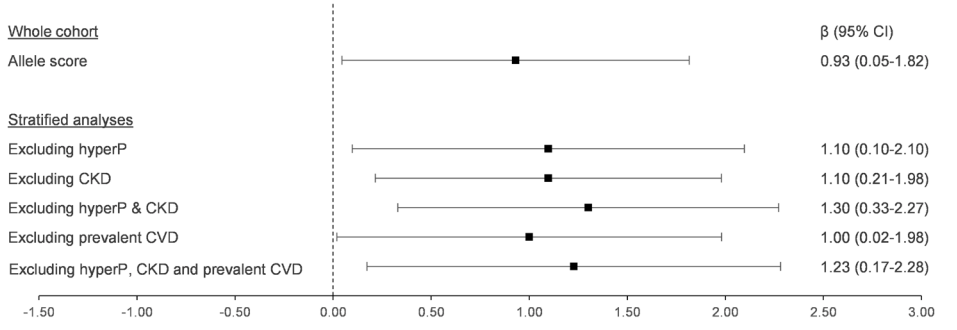
The Sargan tests could not reject the null hypothesis in any case, providing validity of the instruments and an indirect evidence of low heterogeneity among them.

Figure 1. Mendelian Randomization results for serum phosphate and CAC: allelic score method and leave-one-SNP-out approach applied to the whole cohort



Betas were derived from two stage least square for the score as a single instrument and adjusted for age, sexe and 10 principal components. Results are expressed as change in outcome per 1-SD increase in phosphate (0.16 mmol/l=0.49 mg/dL). Leave-one-SNP-out approach: allelic score analyses with the subtraction of one SNP at-a-time. Closest annotated gene is displayed if known to be associated with (or possible related to) phosphate homeostasis. CAC, coronary artery calcification.

Figure 2. Mendelian Randomization results for serum phosphate and CAC: allelic score method applied in subgroup analyses according to serum phosphate levels, kidney function and prevalent cardiovascular disease



Betas were derived from two stage least square for the score as a single instrument and adjusted for age, sexe and 10 principal components. Results are expressed as change in outcome per 1-SD increase in phosphate (0.16 mmol/l=0.49 mg/dL). CAC, coronary artery calcification. HyperP, hyperphosphatemia, defined as a phosphate level > 1.45 mmol/L (=4.5 mg/dL). CKD, chronic kidney disease, defined as a glomerular filtration rate < 60 mL/min/1.73 m2. Prevalent CVD, prevalent cardiovascular disease, defined as prevalent myocardial infarction, revascularization, stroke and heart failure.

EUR-score

Supplementary Table 6 shows that restriction to EUR-score did not attenuate the results of genetically predicted phosphate and CAC (*β*: 1.04, 95 CI: 0.07-2.01, *p*=0.036, n=1693). Results remained significant after excluding hyperphosphatemia (*β*: 1.06, 95 CI: 0.04-2.08, *p*=0.041, n=1659), after excluding hyperphosphatemia and CKD (*β*: 1.30, 95 CI: 0.29-2.30, *p*=0.011, n=1377), and after excluding hyperphosphatemia, CKD and prevalent CVD (*β*: 1.05, 95 CI: 0.08-2.03, *p*=0.035, n=1244)

Assessment of potential horizontal pleiotropy

Concerning MR third assumption (**Supplementary Table 8**), from a frequentist approach the adaptive lasso regression found no evidence of invalidity of instruments; therefore, the inference was similar as that obtained from *tsls*. There was no rejection of the Hansen test, meaning that all SNPs are valid and uncorrelated with error terms.

From a Bayesian approach we applied a method that incorporates pleiotropic effects into a fraction of SNPs and tests whether the association between the genetically predicted exposure and outcome remains. Allowing 49% of SNPs to exert pleiotropic effects and assuring model convergence, we found that genetically predicted phosphate was still associated with CAC: posterior mean: 0.94; 95% *credible* interval (0.13-1.45).

DISCUSSION

Our analyses showed that serum phosphate was strongly associated with CAC, even after excluding subjects with hyperphosphatemia, CKD and prevalent CVD, in a Dutch population-based cohort study. The implementation of MR methods, where genetically predicted phosphate is consistently associated with CAC, strengthens the inference of our observational findings and supports causality. It has been previously shown that hyperphosphatemia or a supraphysiological high phosphate medium is able to induce an osteoblastic transformation of vascular smooth muscle cells with subsequent medial calcification[16,33], characteristic of CKD. Nevertheless, whether normal serum phosphate is associated with intimal calcification has been much less explored[19,21]. Our data demonstrated important sex differences in this association, adding to sex differences previously found concerning serum phosphate and all-cause mortality[46], CVD mortality [14] and atherosclerosis[15], where consistently a stronger (or unique) relation has been described in men.

When analysed as a categorical trait, serum phosphate increases the PR in both sexes for CAC greater than 100, which is considered of moderate risk. In men, serum phosphate increases PR for CAC greater than 300 and greater than 400, which is considered high risk [2]. Remarkably, serum phosphate increases PR for CAC>1000 in both sexes, a category that confers a high mortality risk[34]. To the best of our knowledge, this is the first study to assess the relation between serum phosphate and CAC through MR, by definition less prone to be affected by confounding and reverse causation. Of caution, an important source of bias in MR might be horizontal pleiotropy[47]. Nevertheless, we applied elaborated regression models that assess or allow pleiotropy[42,45]. The persistence of similar results to standard *tsls*, through lasso regression, and the obtainment of significant results despite allowing almost half of the SNPs to exert pleiotropic effects, through Bayesian modelling, confirm the robustness of our findings.

The association between serum phosphate and CAC after restriction of MR to subjects without hyperphosphatemia, CKD and prevalent CVD, supports that increasing (but within normal range) serum phosphate in the general population without clinical CVD is a pathogenic factor for increasing the CAC burden. This finding challenges the concept that only severe hyperphosphatemia, in the uremic context of CKD, is associated with CAC. More importantly, it might provide an explanation for the emerging epidemiologic associations of serum phosphate and increased mortality and CV events in cohorts[46] with mostly normal serum phosphate; and to CVD mortality and atherosclerosis in men with strict normal serum phosphate[14,15]. If these associations are causal there must be an underlying mechanism. CAC induction by an increasing, yet normal, serum phosphate might be one of these mechanisms.

The approach of leaving-one-out SNP has recently been acknowledged as a robust penalization method to test validity in MR[41]. We found that results were not significant when specific SNPs were omitted from the score, one-at-a-time:

a) rs1697421: its omission results in the nullification of the association. This SNP is intergenic but its positional candidate gene is *ALPL*, which encodes for tissue-nonspecific ALP. ALP was not associated with CAC in our population study; but it hydrolyzes PPi into phosphate. PPi is one of the most potent calcification inhibitors[36]. The condition where a SNP affects the outcome through a pathway affected by the risk factor of interest is termed vertical pleiotropy and does not invalidate MR findings. If this SNP influences ALP and its downstream activity/levels, PPi and phosphate, it will correspond to *mediation* of the effect[47].

b) rs2970818: this SNP is also intergenic but one of the positional candidate genes is *FGF23,* which encodes for a key hormone in phosphate homeostasis through increased renal phosphate excretion[18]. In contrast to observational studies linking higher FGF23 levels to arterial calcification, research at the cellular level has shown that FGF23 inhibits osteoblastic differentiation of vascular cells -partially through α-Klotho actions[17,18]. Therefore, horizontal pleiotropy is unlikely.

c) rs35186465: There are not any known phosphate-related genes annotated to this SNP.

Therefore, the association of genetically predicted phosphate with CAC is explained mostly by the contribution of three SNPs from the allele score located in chromosomes 1, 12 and 17. Though a role from several SNPs located throughout the genome improves the validity from MR[43], it seems that rs1697421-near *ALPL*- plays a key role.

Besides FGF23 and α-Klotho, serum phosphate is regulated by $1,25(OH)_2D_3$ and PTH levels. Both $1,25(OH)_2D_3$ and PTH are positively associated with CAC. The significant results from MR analyses decrease their likelihood as confounders. Nevertheless, as PTH has been related to arterial calcification even at normal levels and PTH increases with increased serum phosphate, our data cannot rule out a role of PTH on CAC.

Two main pathways of phosphate-induced calcification have been described in the coronary bed: a) a passive deposition of calcium and phosphate, strongly regulated by ALP-PPi-P, and b) an active process of *osteoblastic differentiation* of vascular pericytes and calcifying vascular cells, able to synthesize matrix vesicles, which start

the mineralization process. Current evidence has shown that ALP, PPi and phosphate are present in matrix vesicles surfaces of atherosclerotic plaques, linking closely both mechanisms of calcification in CAC and potentially providing a biological explanation for our results[4].

Although a RCS model did not find evidence for a non-linear association, we found an apparent dose-effect relation in phosphate and CAC, with *normal* serum phosphate thresholds of 1.09 mmol/L (3.35 mg/dL) and 1.37 mmol/L (4.24 mg/dL) in men and women. Interestingly, Dhingra et al (Framingham study) described a close cut-off for serum phosphate of 3.5 mg/dL (1.13 mmol/L) above which CVD mortality and morbidity increased[13]. The authors stated that it was not clear whether increasing serum phosphate within normal range was associated with CVD risk. Our data suggests that this question can be answered in a confirmatory way.

We also found that *normal* levels of the calcium*phosphate product were associated with CAC. Recent literature highlights that circulating calciprotein particles, composed of calcium, phosphate and calcification inhibitors such as fetuin A, are crucial in calcification and that its composition dictates whether pathologic mineralization is inhibited or not [48]. The calcium*phosphate product within calciprotein particles has been identified as the culprit in this process. Similar to serum phosphate, it might be that a normal product in serum does not reflect a safe product at the cellular level.

The stronger associations observed in men are consistent to previous research on serum phosphate and CVD (atherosclerosis[15], CV event rates[46] and CVD mortality[14,46]). These results are unexpected, especially because women have higher serum phosphate and because the protective effect of 17*β*-estradiol in arterial calcification[49] is predominant in premenopausal women. We can only speculate whether the association between calcium and phosphate levels plays a role in the sex difference as we (and others) have found an inverse relation between serum phosphate and ionized calcium in women but not in men, and an inverse relation between them seems necessary to keep a constant calcium*phosphate product in serum[50,51]. It is important to add that phosphate intake has also been related to arterial calcification, as an abrupt postprandial phosphate increase suffices to initiate mineralization within seconds and to decrease α-klotho expression[52].

This study has several limitations. We had a small sample size and no measurements of $1,25(OH)_2D_3$, FGF23 and PTH levels, nor information on phosphate intake. CAC and ALP were not determined simultaneously. Several tests were not suitable due to our one-sample MR. Moreover, the Rotterdam Study is a Dutch population-based cohort study, precluding inference to other populations or ethnic groups. Lastly, we did not have prospective data on CAC and coronary events, which refrains us from drawing conclusions

about the clinical implications of our findings. But there are several strengths, especially concerning the results from MR, that provides a formal test of causality provided the assumptions are fulfilled. Results from F-statistics strongly suggest that our results are not affected by weak-instrument bias. We were able to perform important stratified and subgroup analyses and to test instruments validity.

To conclude, we hereby provide both frequentist and Bayesian evidence from MR approach that normal phosphate is a causative factor in CAC in the general population without hyperphosphatemia, without prevalent CVD and with normal kidney function. We add more evidence to support the concept of phosphotoxicity and our results call for a review of the current normal serum phosphate range[9]. We agree with the European Food and Safety Agency[53] that more research is needed to study the relationships between dietary intake of phosphate and serum phosphate levels and adverse health outcomes. Public health policies might be needed to decrease phosphate intake, due to the growing evidence of phosphate as a continuous risk factor for adverse outcomes such as atherosclerosis, CVD mortality and now, CAC. Further research should focus in unveiling the underlying mechanisms of the detrimental effects of phosphate in human health and to establish a threshold above which phosphate must be considered harmful for men and women[54], especially because a large fraction of the population appears to be exposed to non-safe P levels.

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Data can be obtained upon request. Requests should be directed towards the management team of the Rotterdam Study (datamanagement.ergo@erasmusmc.nl), which has a protocol for approving data requests. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Supplementary data can be accessed through the following online resources
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9375490/
https://www.ahajournals.org/doi/suppl/10.1161/JAHA.121.023024

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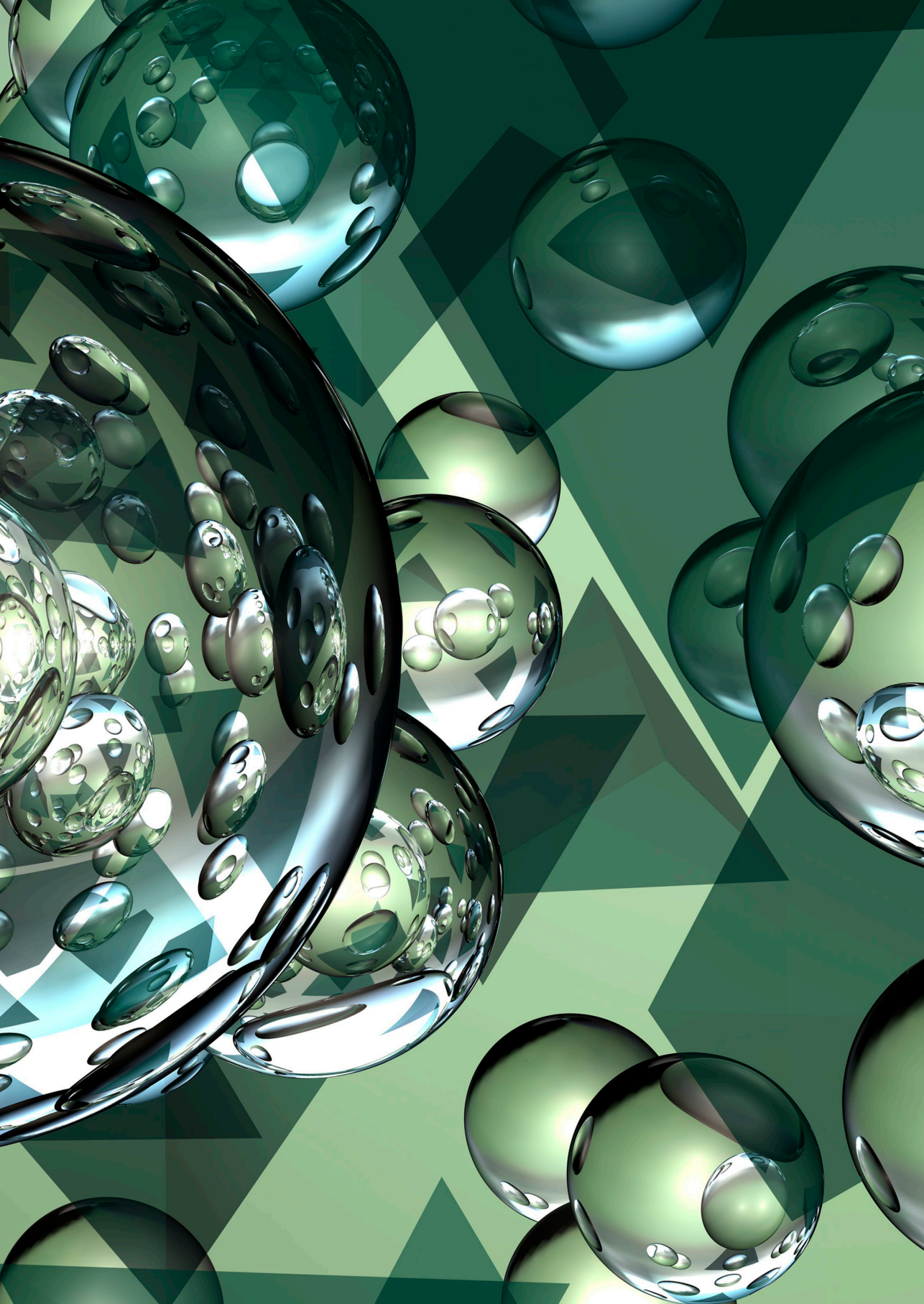
PART V

General Discussion



General discussion

11

The clinical consequences of phosphate disturbances are underappreciated. Although the deleterious effects of hyperphosphatemia in chronic kidney disease are well recognized and attention is paid to hypophosphatemia in acute settings e.g., diabetic ketoacidosis and refeeding syndrome, diseases associated with chronic hypophosphatemia and phosphate disturbances in the general population are less known among health care professionals. The discovery of fibroblast growth factor 23 (FGF23), its role in phosphate homeostasis and the anti-FGF23 antibody burosumab, has led to an increase in research on FGF23-mediated hypophosphatemia and a growing number of studies focus on the adverse effects of increasing yet normal serum phosphate in the general population[1-4].

The aim of this thesis was to extend the knowledge on phosphate homeostasis and on causes and clinical consequences of phosphate disturbances. To achieve this, sex- and age-related differences in serum phosphate, and associations between serum phosphate and BMI and between serum phosphate and diuretic use were analyzed in the general population. Moreover, known causes of hypophosphatemia, including tumor-induced osteomalacia (TIO) and X-linked hypophosphatemia (XLH), were studied, as well as Cushing's syndrome, a disease that is not commonly known to be associated with phosphate disturbances. Lastly, clinical consequences of phosphate disturbances were examined by studying disease characteristics of XLH patients and by exploring the causality of the association between serum phosphate and coronary artery calcification (CAC).

SEX- AND AGE-RELATED DIFFERENCES IN SERUM PHOSPHATE IN THE GENERAL POPULATION

The normal range for serum phosphate is age-specific in infants but age-related changes in serum phosphate later in life have been poorly studied[5]. Moreover, several studies have reported a sex difference in serum phosphate concentrations and an increasing number of studies find sex differences in associations between serum phosphate and morbidity, such as coronary artery calcification, and mortality[4,6-8]. For this reason, we studied age- and sex-related differences in serum phosphate concentrations in the general population in the Rotterdam Study (RS) and UK Biobank (UKBB) (**Chapter 2**). In addition, we studied the influence of estradiol and testosterone on these sex differences in the RS (**Chapter 3**). Our analyses consistently showed that women have higher serum phosphate concentrations than men. This difference becomes more pronounced after menopause but is present at all ages above 45 years. Currently, hypophosphatemia is defined as a serum phosphate concentration below 0.8 mmol/L (2.5 mg/dL) and hyperphosphatemia is defined as a serum phosphate concentration above 1.45 mmol/L (4.5mg/dL)[5,9]. These definitions are identical for both sexes. From our analyses we conclude that it would be more appropriate to set the threshold for

hypophosphatemia at 0.75 mmol/L in men and 0.85 mmol/L in women. The threshold for hyperphosphatemia should be set at 1.35 mmol/L in men and 1.45 mmol/L in women. Future studies on associations between serum phosphate and adverse outcomes should incorporate these thresholds when studying sex-differences. The differences between sexes and the fact that postmenopausal women have higher serum phosphate concentrations than premenopausal women suggest that sex hormones play a role. The results from **Chapter 3** show that both serum estradiol and serum testosterone are inversely associated with serum phosphate in both sexes. Interestingly, the association between serum testosterone and serum phosphate was stronger in men than in women and sex differences in serum phosphate concentration decreased after adjusting for serum testosterone. Moreover, serum testosterone was inversely correlated with serum phosphate in postmenopausal women but not in premenopausal women, suggesting that the influence of serum testosterone on serum phosphate is different between sexes and becomes evident during menopause in women. It remains to be investigated whether only testosterone and not estradiol plays a role in the age-related sex differences in serum phosphate, because testosterone can be converted into estradiol through aromatase, a cytochrome P450 enzyme[10]. Moreover, it is known that estradiol can increase renal phosphate excretion independently from PTH[11], while there are not many studies on the influence of testosterone on phosphate handling[12]. Meng *et al*. hypothesized that testosterone may influence serum phosphate through its effect on bone turnover but there may be also unrecognized effects on renal or gastrointestinal phosphate handling[12]. Future studies in transgender people could aid in understanding the influence of serum testosterone and estradiol, and changes in these concentrations, on serum phosphate.

SERUM PHOSPHATE IN RELATION TO BMI AND DIURETICS USE IN THE GENERAL POPULATION

Fibroblast growth factor (FGF)23, parathyroid hormone (PTH) and 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$) are the three major regulators of serum phosphate concentrations, but there are other hormones that are known to affect serum phosphate e.g., estrogens, glucocorticoids and thyroid hormones [13,14]. Phosphate homeostasis is not yet completely understood and other factors are currently being investigated. One of them is adiposity, which can be quantified using the body mass index (BMI). Inverse associations between serum phosphate and BMI and between serum phosphate and other measures of adiposity have been described in specific patient populations but studies in the general population showed inconclusive results[3,15-19]. Moreover, it was unknown whether these associations are causal and if so, whether serum phosphate influences adiposity or adiposity influences serum phosphate concentrations. For this reason, we performed

a Mendelian randomization (MR) analysis in the RS (**Chapter 4**). We found that serum phosphate was inversely associated with BMI and fat percentage, with a stronger effect in females than in males. MR results suggested a causal effect of BMI on serum phosphate, but not vice versa. Interestingly, results were attenuated after adjustment for leptin. Leptin is derived from white adipose tissue and is strongly associated with adiposity[20]. Mice studies have shown that leptin stimulates FGF23 expression in bone[21]. Consistently, several recent studies have reported associations between BMI and other measures of adiposity, and FGF23[22-24]. Thus, leptin may affect serum phosphate concentrations through FGF23. Another proposed pathway includes 25-hydroxyvitamin-D (25(OH)D. A recent MR study found that the previously reported inverse association between BMI and 25(OH)D is in fact due to a causal effect of BMI on 25(OH)D concentrations. Possible explanations for the effect of BMI on 25(OH)D concentrations include: dilution due to greater volume of distribution; elevated PTH concentrations in obesity and vitamin D deficiency and differences in lifestyle in obese individuals[25]. Phosphate absorption in the intestine is influenced by $1,25(OH)_2D$, which is synthesized from 25(OH)D through the enzyme 1α-hydroxylase. Hence, higher BMI could lower phosphate absorption in the intestine by lowering 25(OH)D concentrations. We controlled our MR analyses for genetically determined vitamin D but this did not change our estimates, most likely because $1,25(OH)_2D$ influences phosphate homeostasis in considerable amount only at the extremes of its concentrations[26]. Given the results from **Chapter 3** we also considered the influence of serum testosterone and estradiol on the association between BMI and serum phosphate, but adjustments for these sex hormones did not change the association in either sex. Our findings imply that adiposity plays a role in phosphate homeostasis and should therefore be taken into account when considering associations of serum phosphate with clinical outcomes. The incidence and degree of hypophosphatemia and its consequences in obesity will need to be studied further.

When studying phosphate homeostasis and phosphate disturbances in humans, exogenous factors such as medication use should not be overlooked. Several antiretroviral, chemotherapeutic and antiepileptic drugs can cause generalized proximal tubulopathy of the kidney, resulting in renal phosphate wasting and hypophosphatemia but also other electrolyte disturbances[27]. Interestingly, several studies have stated that thiazide and loop diuretics are among the most common drugs that have been linked to hypophosphatemia, but this has only been studied in specific patient groups, including hospitalized patients, patients with congestive heart failure or patients with other electrolyte disturbances[28-30]. For this reason, we studied the prevalence of hypophosphatemia in loop and thiazide diuretic users in the RS and in UK Biobank (UKBB) (**Chapter 5**) We found that thiazide users indeed have lower serum phosphate concentrations than thiazide non-users and that hypophosphatemia was more prevalent in female thiazide diuretic users than in female non-users. Loop diuretics were associated

with lower serum phosphate concentrations only in females, not in males, and we found no association with the prevalence of hypophosphatemia in either sex. We observed that the association between thiazide diuretic use and serum phosphate concentration was attenuated after adjustment for BMI in males, while in females the association even lost its significance. This, together with our findings from the above mentioned Mendelian randomization study, implies that hypophosphatemia is more prevalent in thiazide diuretic users partly because users have higher BMI than non-users. Health care professionals who evaluate patients with unexplained hypophosphatemia could therefore consider to pause thiazide diuretics. Moreover, **Chapter 5** also describes the potential role of potassium in phosphate homeostasis. In the RS, thiazide diuretic users had lower serum potassium concentrations than non-users and the associations between thiazide diuretic use and serum phosphate in males lost significance after adjustment for potassium. A study in rats showed that potassium deficiency is associated with a decrease in the sodium phosphate transporters type IIc in the proximal tubule in the kidney[31]. Potassium supplementation has been found to decrease FGF23 and increase serum phosphate and the tubular maximum reabsorption rate of phosphate (TmP/GFR)[32]. Thus, it is possible that drug-induced decreases in serum potassium may influence serum phosphate.

There are a few considerations to be taken into account when interpreting the results from these studies. In the RS, there is no availability of serum FGF23, 1,25$(OH)_2$D nor parathyroid hormone (PTH) concentrations, which are considered to be the major players in phosphate homeostasis. PTH decreases renal absorption of phosphate resulting in increased phosphate excretion[13,14]. We were not able to study the role of PTH on sexual dimorphism in serum phosphate. Recent studies have found an association between PTH and BMI and it has been proposed that 25(OH)D is a mediator of this association[33]. However, other studies showed that increased PTH concentrations in obesity is independent of vitamin D and that leptin increases parathyroid cell mass [20]. The fact that obesity is associated with increased PTH concentrations could partly explain the association between BMI and phosphate. Unfortunately, we had no availability of PTH concentrations.

In addition, the RS is a study of mainly European Caucasians, which precludes us from drawing conclusion on (causal) associations in other populations. Moreover, the RS is a study of elderly males and females. There is more and more evidence that increasing age comes with changes phosphate homeostasis[7,8,34]. A recent study in mice showed that aging female mice upregulate FGF23 to a greater degree during a phosphate challenge than young female mice and male mice. Whether humans show the same age- and sex-related differences remains to be investigated.

CAUSES OF HYPOPHOSPHATEMIA

Hypophosphatemia can be caused by increased renal excretion of phosphate, which can be FGF23-mediated or non-FGF23-mediated. A rare but debilitating cause of FGF23-mediated hypophosphatemia is tumor-induced osteomalacia. This disease is caused by an FGF23-producing tumor which arises from mesenchymal tissue. To better characterize the clinical features of TIO, we performed a systematic clinical review of all published cases of TIO as reported in **Chapter 6**. We found that TIO can develop at any age and tumors can be localized anywhere in the body. Besides hypophosphatemia, low or inappropriately normal $1,25(OH)_2D$ and high FGF23 concentrations, we also found that serum FGF23 was related to tumor size. Alarmingly, many patients had skeletal involvement demonstrated by the high prevalence of fractures and decreased bone mineral density (BMD), which is in agreement with a recent study that reported impairment of bone architecture and strength in TIO patients[35].

We found that the diagnostic delay was longer than 2 years in more than 80% of published cases. Despite the availability of advanced imaging techniques, diagnostic delay has not decreased in the last decade. Moreover, Feng et al. reported an initial misdiagnosis rate of 95.1% in TIO patients. The most frequent initial diagnoses included intervertebral disc herniation, spondylarthritis and osteoporosis[36]. These findings support the need for increased awareness of TIO among health care professionals including endocrinologists but also rheumatologists, neurologists and orthopedic surgeons. The only curative treatment is complete resection of the tumor. The majority of patients can be cured with surgical treatment alone[37]. However, the causative tumor cannot always be identified or (completely) removed[38]. The anti-FGF23 antibody burosumab is increasingly being administered to TIO patients and this results in an increase in serum phosphate and improvement of osteomalacia[39]. The results from **Chapter 6** should be considered whilst taking some limitations of the study design into account. This systematic review only included cases with a reported tumor, while sometimes a causative tumor cannot be identified despite exhaustive investigation[38,40,41]. This review may therefore over represent the less challenging cases of TIO. Second, all data was extracted from case reports and case series, resulting in missing data and variability in diagnostics and treatment.

In **Chapter 7** we present the prevalence of hypophosphatemia in patients with Cushing's syndrome (CS) and the association between the degree of hypercortisolism and serum phosphate. We found that 16% of patients with CS had hypophosphatemia, which is four to six times higher than reported in the general population. Urinary free cortisol excretion, a marker of the degree of hypercortisolism, was inversely associated with serum phosphate. Moreover, serum phosphate concentrations increased after remission

of CS. Glucocorticoids are among the drugs that are most commonly associated with hypophosphatemia in hospitalized patients, but much less is known about serum phosphate concentrations in CS[29]. To the best of our knowledge, the study in **Chapter 7** is the largest to date on serum phosphate concentrations in CS patients. In **Chapter 7** we propose several mechanisms that could explain the effect of glucocorticoids on serum phosphate concentrations. A small cohort study by Findling et al. found that the tubular reabsorption rate of phosphate increases after treatment for CS, suggesting that hypercortisolism affects urinary excretion of phosphate, but the pathophysiological mechanism is currently unknown[42]. As described above, there is evidence that serum potassium plays a role in phosphate homeostasis, possibly mediated by FGF23[31,32]. Hypokalemia can occur in any patient with CS due to saturation of the 11β-hydroxysteroid dehydrogenase type 2 enzyme, which converts cortisol into cortisone, leading to activation of mineralocorticoid receptors. However, in this study we did not find a difference in serum potassium between patients with hypophosphatemia and patients without hypophosphatemia. A second potential mechanisms involves calcium and PTH. It has been hypothesized that glucocorticoids affect serum calcium concentrations by inhibiting intestinal absorption of calcium and increasing urinary calcium excretion[42,43]. Taken together, active CS may result in secondary hyperparathyroidism due to decreased serum calcium concentrations, which in turn may stimulate urinary phosphate excretion. Unfortunately, PTH was not routinely measured in our cohort of CS patients.

Another mechanisms that should not be overlooked is the adiposity-phosphate relation mentioned in **Chapter 4**. The majority of CS patients develop obesity and CS treatment lowers BMI[44,45]. The results in **Chapter 7** show that BMI decreased after treatment for CS in our cohort, which indicates that the changes in serum phosphate may be, at least in part, explained by changes in BMI. The study is limited by the retrospective design and the missing data, but it is clear that future studies are essential to understand the role of cortisol in phosphate homeostasis.

In **Chapter 8**, a 48-year-old woman is reported with unexplained mild hypophosphatemia and very high C-terminal FGF23 (cFGF23) concentrations. Genetic testing and extensive diagnostic imaging were conducted to find the cause of the high cFGF23 concentrations. However, her intact FGF23 (iFGF23) concentration was found to be normal and the patient proved to have an iron deficiency. Iron deficiency alters the ratio between intact iFGF23 and cFGF23 by increasing FG23 transcription through erythropoietin (EPO) or HIF1α directly, and increasing cleavage of FGF23, also mediated by EPO[46,47]. Serum FGF23 concentrations can be measured by two different assays. One is the C-terminal assay, which detects both the active intact FGF23 and the C-terminal fragments that are released after cleavage. The second one is the intact FGF23 assay, which only detects the iFGF23[48,49]. Most clinics, including our clinic, use the cFGF23 assay only. The case

in **Chapter 8** illustrates the limitation of using cFGF23 assays without iFGF23 assays in the setting of FGF23-related disturbances such as iron deficiency. Hypophosphatemia with increased cFGF23 concentrations can be wrongly classified as a FGF23-mediated hypophosphatemia because iFGF23 concentrations may be normal. Therefore, other causes of hypophosphatemia will have to be considered. Commonly applied diagnostic algorithms of hypophosphatemia do not include this feature. Clinicians should be aware of FGF23-related disturbances and iron status should be part of the workup of FGF23-mediated hypophosphatemia. Health care institutions may consider to implement iFGF23 assays.

CONSEQUENCES OF PHOSPHATE DISTURBANCES

Hypophosphatemia can cause a variety of problems such as muscle weakness and rickets[50]. The most common monogenetic cause of chronic hypophosphatemia is X-linked hypophosphatemia (XLH) with an estimated prevalence of 1:20,000-60,000[50-52]. **Chapter 9** describes the only Dutch XLH cohort to date. Disease characteristics and complications were studied in 80 patients from 7 academic hospitals who were included in the observational registry for genetic hypophosphatemia and acquired renal phosphate wasting in the Netherlands (ORPHOS-NED). We found that adiposity, bone deformities, osteoarthritis, hearing loss and hyperparathyroidism are highly prevalent in Dutch XLH patients. Moreover, the XLH patients in our study had short stature compared to the general population and the age at start of XLH-related medical treatment was inversely correlated with the height Z-score at inclusion. This finding support the hypothesis that early initiation of XLH-related medical treatment is beneficial for growth in children[53-55]. However, we and others also found that early initiation of medical treatment is correlated with the occurrence of nephrocalcinosis[52,56]. In fact, nephrocalcinosis was detected by renal imaging in 62% of the pediatric population and 33% of the adult population. Nephrocalcinosis can develop into nephrolithiasis which can lead to chronic kidney disease[57]. It is thought to be the result of medical treatment-induced hypercalciuria. Several studies have suggested associations between oral phosphate supplementation and the risk of nephrocalcinosis, but also between active vitamin D therapy and hypercalciuria[56,58]. In our study presented in **Chapter 9**, we found that nephrocalcinosis was not always preceded by hypercalciuria. A previous study on kidney biopsies in children with hypophosphatemic rickets and nephrocalcinosis showed that the renal calcifications were composed exclusively of calcium and phosphate[59]. Therefore, nephrocalcinosis may not be the result of hypercalciuria alone but of a combination of medication-induced hypercalciuria and hyperphosphaturia resulting in calcium-phosphate deposition. Future studies could focus on the urine phosphate-calcium product and its association with nephrocalcinosis.

In addition, we found that many XLH patients were overweight. This is an important finding because higher BMI, as studied in **Chapter 4** and mentioned previously in this chapter, is causally associated with lower serum phosphate concentrations. Moreover, a higher BMI negatively effects gait and the lateral trunk lean in XLH (known as waddling gait)[60]. Although it is recognized that XLH patients are prone to develop obesity, there are currently no XLH-specific recommendations for its prevention and treatment. A recent international guideline for XLH recommended to prevent and treat obesity as in the general population[61]. These recommendations may not be effective in XLH patients because many patients experience pain or discomfort and disability, which can limit their ability to exercise[62-64]. Moreover, mouse models of XLH showed hyperglycemia and hypoinsulinemia, indicating that the mutation in the *Phex* gene, causative for XLH, also affects glucose and insulin metabolism[65]. Thus, future research on prevention and treatment of obesity in XLH is warranted.

The other side of the abnormal serum phosphate spectrum is hyperphosphatemia, which can cause calcifications. Patients with the monogenetic disease familial tumoral calcinosis present with hyperphosphatemia and ectopic calcifications[66]. Previous studies have found that increasing serum phosphate is associated with vascular calcifications and increased mortality in patients with chronic kidney disease (CKD)[67] but there is also evidence that higher serum phosphate is associated with coronary artery calcification (CAC) in subjects with normal renal function[2]. In **Chapter 10** we studied the association between serum phosphate and CAC in the RS and we applied Mendelian Randomization to examine whether these associations were causal. We found that serum phosphate was associated with CAC in the general population. From Mendelian Randomization analyses we could conclude that these associations are most likely causal. Restricting our analyses to participants without hyperphosphatemia did not change the results, indicating that a higher serum phosphate, even within the normal range, causes coronary artery calcification. Phosphate can induce calcification through passive deposition of calcium and phosphate in the coronary bed. It can also lead to ossification, by osteoblastic differentiation of vascular pericytes and calcifying vascular cells that are able to synthesize matrix vesicles, starting the process of mineralization[68]. Moreover, we found marked sex-differences in the association between serum phosphate and CAC. The association was significant in men with and without impaired kidney function, while in women the association was constrained to those with normal kidney function. In addition, analyses in quintiles showed that men with serum phosphate above 1.09 mmol displayed a significant trend for higher CAC, while for women this threshold was set at 1.37 mmol/L. Lastly, when we analyzed CAC as a categorical trait, using different cut-offs for CAC scores, we found that serum phosphate was associated with a higher prevalence ratio of CAC scores across all severities, while in women serum phosphate was associated with a higher prevalence ratio of very high CAC scores only. These findings indicate that,

although women have higher serum phosphate concentrations than men, deleterious effects of higher phosphate are more pronounced in men. These findings support our previous conclusion in **Chapter 2** that there is a need for a sex-specific reference range for serum phosphate, which should include an upper limit of normal that is lower than what is currently accepted in males.

CLINICAL IMPLICATIONS AND FUTURE RESEARCH

The studies presented in this thesis aimed to extend the knowledge on phosphate homeostasis and on causes and clinical consequences of phosphate disturbances. We showed that women have higher serum phosphate concentrations than men. This difference becomes more pronounced after menopause but is present at all ages above 45 years. Moreover, the population range for serum phosphate is higher in women than in men. Given the sex-differences in the associations of serum phosphate with morbidity like CAC and mortality, this finding seems to be highly relevant and sex-specific references ranges for serum phosphate are warranted. We propose to introduce sex-specific reference ranges for serum phosphate in adults older than 45 years: e.g., 0.75 – 1.35 mmol/L in males and 0.85 – 1.45 mmol/L in females. Our findings are important for several reasons: future association analyses of serum phosphate with clinical outcomes should take into account sex and age; laboratories of health care institutions should consider age- and sex-specific reference ranges; and clinicians who treat patients with phosphate disturbances should be aware of sex-differences in serum phosphate.

Our diet is the primary source of phosphate and phosphate is present in most types of foods. Phosphate-rich food groups are dairy products, meat and fish. Over time, the phosphate load of the Western diet has increased due to phosphate-containing additives in processed foods, but the phosphate content of a product is generally not provided[69,70]. In 1976, Marshall et al. reported that the total content of phosphate in human plasma is comprised of 72% organic phosphate and 28% inorganic phosphate[71]. It is currently unknown whether the changes in diet have changed the composition of the total phosphate content in human plasma and a relation with FGF23. It has been estimated that the recommended daily allowance of dietary phosphate is exceeded by a factor of two in the American diet[72]. It remains to be investigated whether a phosphate-rich diet increases the risk for adverse outcomes such as CAC. It has been shown that serum phosphate has a circadian rhythm, with the lowest phosphate concentrations occurring in the morning, followed by a mild peak and drop during the day and a larger peak during the night[73]. This circadian rhythm is influenced by dietary phosphate[74]. Health care professionals should be aware of this circadian rhythm when diagnosing and treating patients with phosphate related disturbances. Whether there are sex-

differences in this circadian rhythm and in an effect of dietary phosphate thereon is currently unknown. In the RS blood samples were taken following fasting while in UKBB blood samples were taken regardless of fasting status. However similar sex differences in serum phosphate concentrations were seen in both studies. Future research on serum phosphate should take in account this circadian rhythm as it may influence any associations that are being investigated.

This thesis also highlights the limitation of the use of cFGF23 assays in the diagnostic work-up of hypophosphatemia, because it does not provide information on the presence of FGF23-related disturbances. Indeed, measurement of iFGF23 has been found to be superior to cFGF23 in making a diagnosis of FGF23-mediated hypophosphatemia[75]. Therefore, health care professionals should be aware of the differences between iFGF23 and cFGF23 assays and health care institutions should consider to change to or at least implement iFGF23 assays.

The fact that adiposity is causally associated with lower serum phosphate concentrations should be taken in account in future studies on associations of serum phosphate with clinical outcomes. This finding may also be important for health care professionals who treat XLH patients, given the fact that obesity is highly prevalent in XLH patients. It remains to be elucidated whether higher BMI is also associated with lower serum phosphate concentrations in XLH. Furthermore, it will be interesting to study the effect of weight loss through glucacon like peptide-1 (GLP-1) analogues on serum phosphate concentrations. Bariatric surgery is an effective treatment for obesity but it is associated with secondary hyperparathyroidism, which is thought to be a consequence of calcium malabsorption[76]. GLP-1 analogues have been found to effective in weight loss without disruption of intestinal absorption[77].

We found that thiazide users have lower serum phosphate concentrations than thiazide non-users and that hypophosphatemia was more prevalent in thiazide users. Adiposity may in part explain the association between thiazide diuretic use and serum phosphate concentration. Interestingly, thiazide diuretics are being prescribed to patients with XLH to decrease drug-induced urinary calcium excretion and to prevent nephrocalcinosis[78]. In a small cohort study in pediatric XLH patients, thiazide diuretic use led to a decrease in urinary calcium excretion, but it also decreased serum phosphate[79]. These findings call for a larger study on the effect of thiazide diuretic use on serum phosphate in XLH. Taken together, caution is warranted when prescribing thiazide diuretics to XLH patients or patients with unexplained hypophosphatemia. Our finding that loop diuretic use was not associated with hypophosphatemia contradicts a previous study stating that loop diuretics should be considered as one of the most common drugs associated with hypophosphatemia[27].

In our search for regulators of serum phosphate, we found that the prevalence of hypophosphatemia in CS patients was four to six times higher than reported in the general population and that the severity of the hypercortisolism in these patients was inversely associated with serum phosphate. Many CS patients experience muscle weakness due to glucocorticoid induced myopathy, caused by altered protein metabolism. Hypophosphatemia can cause an decrease in muscle ATP synthesis resulting in worsening of muscle weakness in CS patients[80,81] Moreover, bone health in CS patients, which is already impaired by glucocorticoid-induced low bone mineral density, may further deteriorate due to osteomalacia. It should be noted that we conducted a retrospective study with serum phosphate measured at random and there was no availability of PTH or FGF23. To validate our findings, future prospective studies in CS should include measurement of serum phosphate, PTH, FG23, TmP/GFR, cortisoluria and BMI at baseline and after curative treatment at predefined intervals. When our findings are replicated, it will be important to study the effect of phosphate supplementation on the severity of fatigue, muscle weakness and also bone health.

Lastly, to improve knowledge on phosphate homeostasis it will be essential to conduct new and larger genome wide association studies (GWAS) on serum phosphate. Currently, two GWASs on serum phosphate have been published and the Single Nucleotide Polymorphisms (SNP) that these studies identified were used in the Mendelian randomization studies in this thesis[82,83]. Since the publication of these two GWASs, larger datasets such as UK Biobank have become available and options for downstream analysis have increased. A new GWAS on serum phosphate, and also on FGF23, can aid in further understanding biological en pathophysiological mechanisms

In conclusion, our knowledge on phosphate homeostasis is expanding and the consequences of phosphate disturbances are increasingly recognized. Still, many areas of phosphate research are untouched or require additional investigation.

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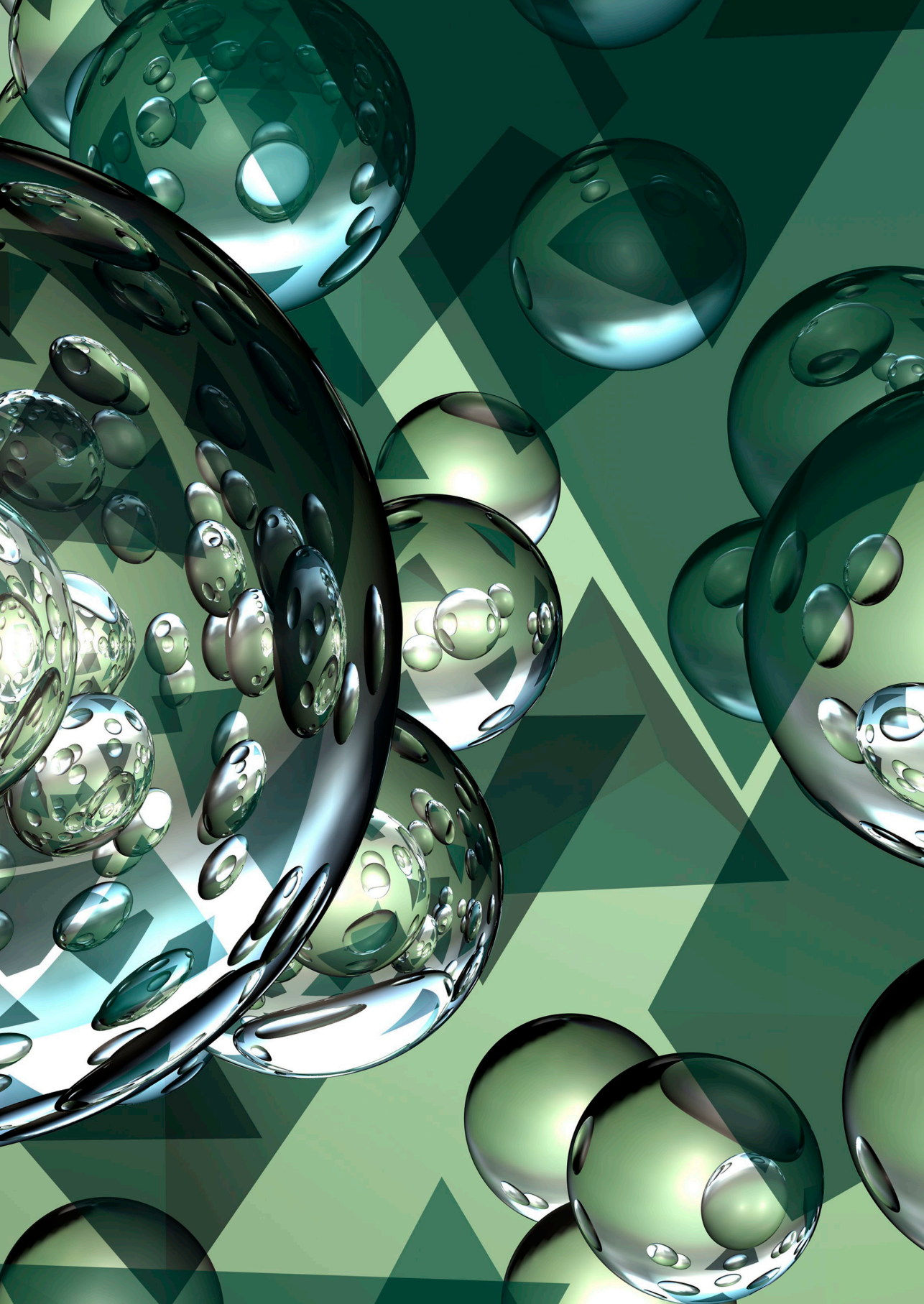

Summary
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SUMMARY

Phosphorus has many functions in the human body. The phosphorus content is comprised of organic and inorganic phosphate. In this thesis, inorganic phosphate is discussed because it can be measured in bodily fluids. Most of the inorganic phosphate in humans (85%) is stored as hydroxyapatite in bone and teeth. Approximately 14% of the total inorganic phosphate content can be found in the intracellular compartment and the remaining 1% is present in the extracellular compartment. Phosphate is important for cell metabolism, muscle function and bone mineralization. The serum phosphate concentration is regulated by the actions of by 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$), also known as calcitriol, parathyroid hormone (PTH) and fibroblast growth factor (FGF)23. Low serum phosphate, named hypophosphatemia, causes rickets with the development of bone deformities in children and osteomalacia in adults. Chronic hypophosphatemia can be caused by (mono)genetic disorders but it can also develop during the course of life e.g., due to an FGF23-producing tumor or due to medication use. High serum phosphate, named hyperphosphatemia, can lead to the formation of ectopic calcifications. Hyperphosphatemia can be caused by a (mono)genetic disease or by impaired urinary phosphate excretion in the setting of chronic kidney disease. Despite numerous studies, phosphate homeostasis is still incompletely understood, and knowledge of causes and consequences of phosphate disturbances is still often poor among health care professionals.

The aim of our research in this thesis is to extend the knowledge on phosphate homeostasis and on causes and clinical consequences of phosphate disturbances.

Part I: Sex and age related differences in serum phosphate in the general population
In general, a fasting serum phosphate concentration in adults is considered normal when it lies between 0.80 mmol/L and 1.45 mmol/L. It is well known that serum phosphate concentrations are higher in infants and decrease during childhood and adolescence. Much less is known about potential age-related changes in serum phosphate during adulthood and whether these are sex-related.

In **Chapter 2** the sex- and age-related differences were studied in two population-based cohorts, namely the Rotterdam Study (RS) and UK Biobank (UKBB). This study consistently showed that adult women have higher serum phosphate levels than men. Moreover, while in both elderly men and women serum phosphate concentrations decrease with age, women appear to go through an initial increase in serum phosphate around menopause. The population range for serum phosphate is higher in women than in men resulting in a higher prevalence of hypophosphatemia in men and a higher prevalence of hyperphosphatemia in women. Given the sex-differences in the associations of

serum phosphate with morbidity and mortality, sex-specific references ranges for serum phosphate are warranted. We propose to introduce sex-specific reference ranges for serum phosphate above the age of 45 years, e.g., 0.75 – 1.35 mmol/L in males and 0.85 – 1.45 mmol/L in females.

In **Chapter 3** the sexual dimorphism in serum phosphate and calcium was further investigated in RS and especially the potential role of body mass index (BMI), kidney function, smoking and sex hormones, vitamin D and alkaline phosphatase. Serum total calcium concentrations were also higher in women than in men, but to a lesser degree than serum phosphate. BMI, kidney function and smoking nor serum vitamin D and alkaline phosphatase explained the sex-differences in serum phosphate and serum calcium. Serum estradiol but not testosterone was inversely associated with serum calcium while serum testosterone was inversely associated with serum phosphate in both sexes. Similarly, adjustment for serum estradiol diminished sex differences in serum calcium while adjustment for serum testosterone diminished sex differences in serum phosphate. Taken together, these results indicate that serum testosterone may in part explain sex differences in serum phosphate while estradiol could partly explain sex differences in serum calcium.

Part II: Serum phosphate in relation to BMI and diuretics use in the general population
Apart from PTH, FGF23 and $1,25(OH)_2D$ there may be other factors influencing serum phosphate.

Chapter 4 describes the results of a Mendelian randomization (MR) study on the causality of the association between BMI and serum phosphate in RS. Serum phosphate was inversely associated with BMI and fat percentage, with a stronger effect in females than in males. These associations were not influenced by education level, smoking, total calcium, 25-hydroxy-vitamin D, kidney function nor sex hormones. Adjusting for leptin attenuated the relation between BMI and serum phosphate in females only. Bi-directional MR analysis suggested that BMI lowers phosphate but phosphate does not seem to affect BMI.

Thiazide and loop diuretics are considered to be among the most common drugs linked to hypophosphatemia. However, their effects on phosphate handling and prevalence of hypophosphatemia have been mainly studied in specific patient groups e.g., hospitalized patients and patients with other electrolyte disturbances. In **Chapter 5**, the results of our study on the association between loop and thiazide diuretic use and serum phosphate concentration and hypophosphatemia in RS and UKBB are presented. Loop diuretic use was associated with lower serum phosphate concentrations in females but not in males in both cohorts. BMI partly explained the association between loop diuretic

use and serum phosphate in women. Thiazide diuretic use was associated with lower serum phosphate concentrations in both sexes in both cohorts. BMI partly explained the association between thiazide diuretic use and serum phosphate in both sexes, while serum potassium also decreased the association in men. Thiazide diuretic use was associated with an increased risk of hypophosphatemia in women, while results in men were inconclusive. Thiazide diuretic use and increased BMI should be considered as a contributing factor in women with hypophosphatemia.

Part III: Causes of hypophosphatemia

Hypophosphatemia can be caused by a shift of phosphate from the extra- to the intracellular compartment, by impaired intestinal phosphate absorption or intake, or by increased renal excretion of phosphate. Hypophosphatemia resulting from increased renal excretion can be further divided in FGF23-mediated and non-FGF23-mediated hypophosphatemia.

A rare and largely underdiagnosed cause of acquired FGF23-mediated hypophosphatemia is tumor-induced osteomalacia (TIO). This disease leads to rickets in children and osteomalacia in adults, with bone pain, fractures and muscle weakness. The only curative treatment is complete resection of the tumor. When curative resection is not possible, patients can be treated with phosphate and/or active vitamin D supplementation or with the anti-FGF23 antibody burosumab. **Chapter 6** presents the results from a systematic clinical review of published cases of TIO, and describes clinical and biochemical characteristics including bone involvement, tumor localization and treatment of 895 unique TIO cases. TIO can develop at any age and appears to develop more often in males than in females. The majority of patients present with hypophosphatemia and inappropriately low or normal $1,25(OH)_2D$ concentrations. We found a median tumor size of 2.7 cm, but tumor sizes ranged from 0.5 to 25.0 cm. Serum FGF23 was positively correlated with tumor size. In 32% of the cases the tumor was detected by physical examination. Data on bone phenotype confirmed skeletal involvement: 62% of cases with data on bone mineral density had a T-score of the lumbar spine ≤-2.5 (n=61/99) and a fracture was reported in at least 39% of all cases (n=346/895). Diagnostic delay was longer than 2 years in more than 80% of cases. 10% were reported to be malignant at histology. In conclusion, TIO is a debilitating disease characterized by a long diagnostic delay leading to metabolic disturbances and skeletal complications. Increasing awareness of TIO should accelerate detection and reduce metabolic disturbances and skeletal impairment.

Another potential cause of hypophosphatemia is the use of corticosteroids. Little is known about the occurrence of hypophosphatemia in patients with Cushing's syndrome (CS), a disease characterized by hypercortisolism. **Chapter 7** presents the prevalence of

hypophosphatemia in a cohort of patients with CS who were diagnosed and treated at the Erasmus MC. Hypophosphatemia before treatment was present in 16% of the 99 CS patients. In comparison, the prevalence of hypophosphatemia in RS was 2.0-4.2%. Moreover, serum phosphate was related to the degree of hypercortisolism, quantified by the level of urinary free cortisol excretion, and increased after remission of CS. The results in **Chapter 7** show that CS can be a cause of hypophosphatemia, a condition that is currently not incorporated in the differential diagnosis of hypophosphatemia. Potential underlying mechanisms related to urinary phosphate excretion and possibly involving FGF23, BMI and parathyroid hormone levels should be further explored.

In **Chapter 8** the medical history of a 48-year-old women is reported, who had unexplained mild hypophosphatemia and very high cFGF23 concentrations. We conducted extensive diagnostic procedures including advanced imaging and genetic testing, because cFGF23 concentrations kept rising, in search of a growing tumor causing TIO. Circulating FGF23 concentrations in humans can be quantified using two types of FGF23 assays: the C-terminal FGF23 (cFGF23) assay detects both the active full-length FGF23 and the C-terminal fragments that are released after cleavage, while the intact FGF23 (iFGF23) assay detects only the presumed active full-length FGF23. Our patient turned out to have very high cFGF23 concentrations but normal iFGF23 due to an iron deficiency. Iron deficiency alters the iFGF23-to-cFGF23 ratio. It increases *FGF23* expression and simultaneously increases cleavage to retain homeostasis resulting in relatively high cFGF23 concentrations while iFGF23 concentrations remain normal. After initiation of iron treatment, cFGF23 concentrations in the patient strongly decreased. This case report challenges the applicability of cFGF23 assays for the work-up of hypophosphatemia in the setting of FGF23-related disturbances, and emphasizes the need for assessment of iron status in the work-up of FGF23-mediated hypophosphatemia.

Part IV: Consequences of phosphate disturbances

Chronic hypophosphatemia can lead to rickets with the development of bone deformities in children and osteomalacia in children and adults. Hyperphosphatemia on the other hand can cause ectopic calcifications.

The most common monogenetic cause of chronic hypophosphatemia is X-linked hypophosphatemia (XLH). **Chapter 9** describes disease manifestations and complications in a cohort of 80 Dutch XLH patients who were included in the Observational registry for Rare genetic hypoPHOSphatemia and acquired renal phosphate wasting in the NEtherlanDs (ORPHOS-NED). Most patients had a positive family history for XLH. Patients had short stature compared to the general Dutch population and many were overweight. Age at start of XLH-related medical treatment was inversely correlated with height at inclusion. Almost one third of adults had hearing problems. Knee deformities were

present in 75% of patients and osteoarthritis was reported in 51% of patients. Almost half of patients had undergone at least one orthopaedic intervention. Nephrocalcinosis was observed in 62.1% of children and 33.3% of adults. The age at start of XLH-related medication was inversely correlated with the occurrence of nephrocalcinosis. This nationwide study confirms the high prevalence of adiposity, bone deformities, osteoarthritis, hearing loss and nephrocalcinosis in Dutch XLH patients. Early start of XLH-related treatment appears to be beneficial for longitudinal growth but may be a risk factor for the development of nephrocalcinosis.

In **Chapter 10**, the association between serum phosphate and coronary artery calcification (CAC) is investigated within RS. CAC was measured by electron-beam computed tomography. Higher serum phosphate was associated with higher CAC scores, with a stronger effect in men compared to women. Exclusion of participants with hyperphosphatemia, chronic kidney disease and prevalent cardiovascular diseases yielded similar results. MR analyses supported a causal relation, also for serum phosphate and CAC in subjects without hyperphosphatemia, chronic kidney disease and cardiovascular disease. This suggests that higher serum phosphate concentrations, even within the normal range, may be harmful, validating our conclusion from **Chapter 2** that the normal range for serum phosphate in men and women should be reconsidered.

Finally, in **Chapter 11**, the results presented in this thesis are discussed and clinical implications are considered.

SAMENVATTING

Fosfor heeft vele functies in het menselijk lichaam. De totale hoeveelheid fosfor in het lichaam is opgebouwd uit organisch en anorganisch fosfaat. In dit proefschrift is gekeken naar anorganisch fosfaat omdat dit gemeten kan worden in lichaamsvloeistoffen. Het grootste deel van het anorganisch fosfaat in het menselijk lichaam (85%) is opgeslagen in de vorm van hydroxyapatiet in bot en tanden. Ongeveer 14% van de totale hoeveelheid anorganisch fosfaat bevindt zich in het intracellulaire compartiment en de resterende 1% bevindt zich in het extracellulaire compartiment. Fosfaat is belangrijk voor celmetabolisme, spierfunctie en botmineralisatie. De concentratie fosfaat in het bloedserum wordt hoofdzakelijk gereguleerd door 1,25-dihydroxy-vitamine D ($1,25(OH)_2D$), ook wel bekend als calcitriol, parathyreoïd hormoon (PTH) en fibroblast groei factor (FGF)23. Een verstoring in de fosfaatbalans, de homeostase, leidt tot fosfaatstoornissen. Een lage fosfaatconcentratie in het serum, ofwel hypofosfatemie, veroorzaakt rachitis wat kan leiden tot botdeformiteiten in kinderen en osteomalacie in volwassenen. Chronische hypofosfatemie kan veroorzaakt worden door (mono)genetische aandoeningen maar het kan ook op oudere leeftijd ontstaan, bijvoorbeeld door een FGF23-producerende tumor of door het gebruik van bepaalde medicatie. Een hoge fosfaatconcentratie in het serum, ofwel hyperfosfatemie, kan leiden tot het ontstaan van ectopische calcificaties. Hyperfosfatemie kan worden veroorzaakt door een (mono)genetische aandoening of door een verminderde fosfaatexcretie via de urine bij nieraandoeningen. Er wordt veel onderzoek naar gedaan maar de fosfaat homeostase is niet geheel opgehelderd en medisch specialisten zijn onvoldoende bekend met de oorzaken en gevolgen van fosfaatstoornissen.

Het doel van het onderzoek in dit proefschrift is het uitbreiden van de kennis omtrent fosfaat homeostase en oorzaken en gevolgen van fosfaatstoornissen.

Deel I: Geslacht- en leeftijdsverschillen in serum fosfaat in de bevolking

Over het algemeen wordt een fosfaatconcentratie tussen de 0.80 mmol/L en 1.45 mmol/L als normaal geclassificeerd bij volwassenen. Het is bekend dat de fosfaatconcentratie in het serum van een pasgeborene hoger is en lager wordt gedurende de kindertijd en adolescentie. Er is veel minder bekend over de geslacht- en leeftijdsverschillen in de fosfaatconcentratie in serum bij volwassenen.

In **hoofdstuk 2** wordt een onderzoek beschreven naar de geslacht- en leeftijdsverschillen in de fosfaatconcentratie in twee grote cohortstudies, namelijk de Rotterdam studie (RS) en UK Biobank (UKBB). Dit onderzoek laat duidelijk zien dat vrouwen een hogere fosfaat concentratie in serum hebben dan mannen. In zowel oudere mannen als vrouwen daalt de fosfaatconcentratie met de leeftijd maar vrouwen ondergaan een initiële stijging van de fosfaatconcentratie ten tijde van de overgang. De gemiddelde

waarden van de fosfaatconcentratie ligt bij vrouwen hoger dan bij mannen. Met de huidige referentiewaarden lijkt hypofosfatemie vaker voor te komen bij mannen en hyperfosfatemie vaker bij vrouwen. Het is bekend dat er geslachtsverschillen bestaan in de associaties tussen fosfaatconcentratie en morbiditeit en mortaliteit, waardoor dus overwogen moet worden om geslachtsafhankelijke referentiewaarden voor fosfaat te specificeren. Op basis van dit onderzoek stellen wij de volgende geslachtsafhankelijke referentiewaarden voor de fosfaatconcentratie in serum van volwassenen ouder dan 45 jaar voor: 0.75-1.35 mmol/L bij mannen en 0.85-1.45 mmol bij vrouwen.

In **hoofdstuk 3** worden de geslachtsverschillen in fosfaat- en calciumconcentratie verder onderzocht in RS. Hierbij wordt gekeken naar de rol van de body mass index (BMI), de nierfunctie, roken, geslachtshormonen, vitamine D en alkalisch fosfatase. De serum calcium (totaal) concentratie bleek bij vrouwen ook hoger te zijn dan bij mannen maar het verschil was minder groot dan bij de fosfaatconcentratie. De geslachtsverschillen in fosfaat- en calciumconcentratie blijken niet verklaard te worden door verschillen in BMI, nierfunctie, roken, vitamine D of alkalisch fosfatase. Serum estradiol, maar niet testosteron, was negatief geassocieerd met serum calcium, terwijl serum testosteron negatief geassocieerd was met de fosfaatconcentratie bij zowel mannen als vrouwen. De verschillen tussen mannen en vrouwen in calciumconcentratie werden kleiner na adjusteren voor serum estradiol, terwijl de verschillen in serumfosfaat kleiner werden na adjusteren voor serum testosteron. Deze resultaten laten zien dat de verschillen tussen mannen en vrouwen in fosfaatconcentratie deels verklaard kunnen worden door verschillen in serum testosteron terwijl serum estradiol deels de verschillen tussen mannen en vrouwen in calciumconcentratie zou kunnen verklaren.

Deel II: Serum fosfaat in relatie tot BMI en diuretica gebruik in de bevolking

Het is bekend dat PTH, FGF23 en 1,25$(OH)_2$D een belangrijke rol spelen bij de regulatie van de fosfaatconcentratie maar mogelijk zijn er ook andere factoren die invloed hebben op de fosfaatconcentratie. **Hoofdstuk 4** beschrijft de resultaten van een Mendelian randomization (MR) studie naar de causaliteit van de associatie tussen BMI en serum fosfaat in RS. Serum fosfaat was negatief geassocieerd met BMI en vetpercentage, waarbij de associatie sterker was bij vrouwen dan bij mannen. Deze associaties werden niet beïnvloed door opleiding, roken, totaal calcium, 25-hydroxy-vitamine D, nierfunctie of geslachtshormonen. De associatie tussen BMI en fosfaatconcentratie werd minder sterk bij vrouwen na adjusteren voor leptin. Een bi-directionele MR analyse suggereerde dat een hoger BMI leidt tot een lager fosfaat maar dat fosfaat geen effect heeft op BMI.

Thiazide- en lisdiuretica vallen onder de medicijnen die het meest worden geassocieerd met hypofosfatemie. Echter, het effect van deze diuretica op fosfaatregulatie en prevalentie van hypofosfatemie is alleen in specifieke patiëntenpopulaties onderzocht:

patiënten die opgenomen waren in het ziekenhuis en patiënten met bijkomende andere elektrolytstoornissen. In **Hoofdstuk 5** worden de resultaten gepresenteerd van een studie naar de associatie tussen thiazide- en lisdiuretica en serum fosfaat concentratie en hypofosfatemie in RS en UK Biobank. Het gebruik van lisdiuretica was geassocieerd met een lagere fosfaatconcentratie in serum bij vrouwen. Gebruik van thiazidediuretica was geassocieerd met lagere fosfaatconcentraties bij zowel mannen als vrouwen in beide cohorten. De associatie tussen thiazidediuretica gebruik en fosfaatconcentratie bij mannen en vrouwen lijkt ten dele verklaard te kunnen worden door een verschil in BMI, maar de associatie bij mannen werd ook minder sterk na adjusteren voor serum kalium. Thiazidediuretica gebruik was geassocieerd met een hoger risico op hypofosfatemie in vrouwen maar deze resultaten waren niet eenduidig bij mannen. Thiazidediuretica en een verhoogd BMI moeten gezien worden als bijdragende factoren bij vrouwen met onbegrepen hypofosfatemie.

Deel III: Oorzaken van hypofosfatemie

Hypofosfatemie kan veroorzaakt worden door een verplaatsing van fosfaat van het extra- naar het intracellulaire compartiment, door verminderde inname of intestinale absorptie van fosfaat of door een toegenomen renale excretie van fosfaat. Hypofosfatemie welke veroorzaakt wordt door een toegenomen renale excretie kan verder onderverdeeld worden in FGF23-gemedieerde en niet-FGF23-gemedieerde hypofosfatemie.

Tumor geïnduceerde osteomalacie (TIO) is een zeldzame en ondergediagnosticeerde oorzaak van verworven FGF23-gemedieerde hypofosfatemie. Deze aandoening leidt tot rachitis bij kinderen en osteomalacie bij volwassenen, wat gepaard gaat met botpijn, botbreuken en spierzwakte. De enige curatieve behandeling is complete tumorresectie. Als resectie niet mogelijk is kunnen patiënten behandeld worden met fosfaat en/ of actieve vitamine D suppletie. Ook het anti-FGF23 antilichaam burosumab kan aan patiënten met TIO voorgeschreven worden. **Hoofdstuk 6** presenteert de resultaten van een systematische literatuurstudie van gepubliceerde gevallen van TIO. Deze studie beschrijft de biochemische en klinische karakteristieken, zoals botbetrokkenheid, tumorlokalisatie en behandeling, van 895 unieke patiënten met TIO. TIO kan op iedere leeftijd ontstaan en lijkt vaker voor te komen bij mannen dan bij vrouwen. De meerderheid van de patiënten hebben hypofosfatemie en verlaagde of normale $1,25(OH)_2D$ concentraties. De mediane tumor afmeting was 2.7 cm maar de afmetingen varieerden van 0.5 tot 25.0 cm. Serum FGF23 was positief gecorreleerd met de afmeting van de tumor. In 32% van de gevallen kon de tumor gevonden worden bij het lichamelijk onderzoek. In de meeste gevallen was er sprake van bot betrokkenheid: 62% van de patiënten met data over de botdichtheid had een T-score ≤ -2.5 (n=61/99) van de lumbale wervelkolom. Een doorgemaakte fractuur was gerapporteerd in 39% van alle gevallen (n=346/895). Bij ruim 80% van de patiënten duurde het diagnostische proces

meer dan 2 jaar. 10% van de tumoren werd geclassificeerd als maligne bij histologisch onderzoek. Deze studie laat zien dat TIO een invaliderende ziekte is die gekarakteriseerd wordt door een lang diagnostisch proces en die gepaard gaat met metabole afwijkingen en bot gerelateerde complicaties. Het verbeteren van de herkenning van TIO zal leiden tot een verkorting van het diagnostisch proces en minder metabole afwijkingen en bot gerelateerde problematiek.

Een andere mogelijke oorzaak van hypofosfatemie is het gebruik van corticosteroïden. Er is weinig bekend over de prevalentie van hypofosfatemie bij patiënten met het syndroom van Cushing (CS), een ziekte die gekarakteriseerd wordt door hypercortisolisme. **Hoofdstuk 7** beschrijft de prevalentie van hypofosfatemie in een cohort van CS patiënten die gediagnosticeerd en behandeld waren in het Erasmus MC. Voorafgaand aan behandeling was er sprake van hypofosfatemie bij 16% van de 99 CS patiënten. In vergelijking, de prevalentie van hypofosfatemie in RS ligt tussen de 2.0 en 4.2%. De fosfaatconcentratie bleek gerelateerd aan de ernst van het hypercortisolisme, wat gemeten kan worden middels de vrije cortisol excretie in urine. Daarbij was een stijging te zien in de fosfaatconcentratie bij remissie van de CS. De resultaten in **hoofdstuk 7** laten zien dat CS een oorzaak zou kunnen zijn van hypofosfatemie, maar CS wordt op dit moment niet meegenomen in de differentiaal diagnose van hypofosfatemie. Toekomstig onderzoek moet uitwijzen wat het pathofysiologisch mechanisme is en wat de rol is van FGF23, BMI en PTH.

In **hoofdstuk 8** wordt het ziektebeloop beschreven van een 48-jarige vrouw die werd gezien in verband met onbegrepen milde hypofosfatemie en hele hoge cFGF23 concentraties. Omdat de cFGF23 concentratie bleef stijgen werd uitgebreid diagnostisch onderzoek ingezet met onder andere genetische diagnostiek en geavanceerde beeldvorming op zoek naar een groeiende tumor in het kader van TIO. De FGF23 concentratie in het menselijk lichaam kan gemeten worden middels twee verschillende FGF23 assays: de C-terminus FGF23 (cFGF23) assay meet zowel het actieve intacte FGF23 als ook de C-terminus fragmenten die vrijkomen nadat het intacte FGF23 geknipt wordt; de intacte FGF23 (iFGF23) assay detecteert alleen het actieve intacte FGF23. De patiënte in **hoofdstuk 8** had hele hoge cFGF23 concentraties maar een normaal iFGF23 door een ijzerdeficiëntie. IJzerdeficiëntie heeft een effect op de iFGF23/cFGF23 ratio. Het leidt tot een verhoogde *FGF23* expressie met gelijktijdig een toename in de afbraak van FGF23 waardoor de homeostase wordt behouden maar waarbij er relatief hoge cFGF23 concentraties worden gemeten bij een normaal iFGF23. Na start van behandeling met ijzersuppletie daalden de cFGF23 concentraties. Dit ziektebeloop toont de beperkingen van het gebruik van cFGF23 bij de diagnostiek naar hypofosfatemie ten tijde van FGF23-gerelateerde stoornissen. Een bepaling van de ijzerstatus moet meegenomen worden in de diagnostiek naar FGF23-gemedieerde hypofosfatemie.

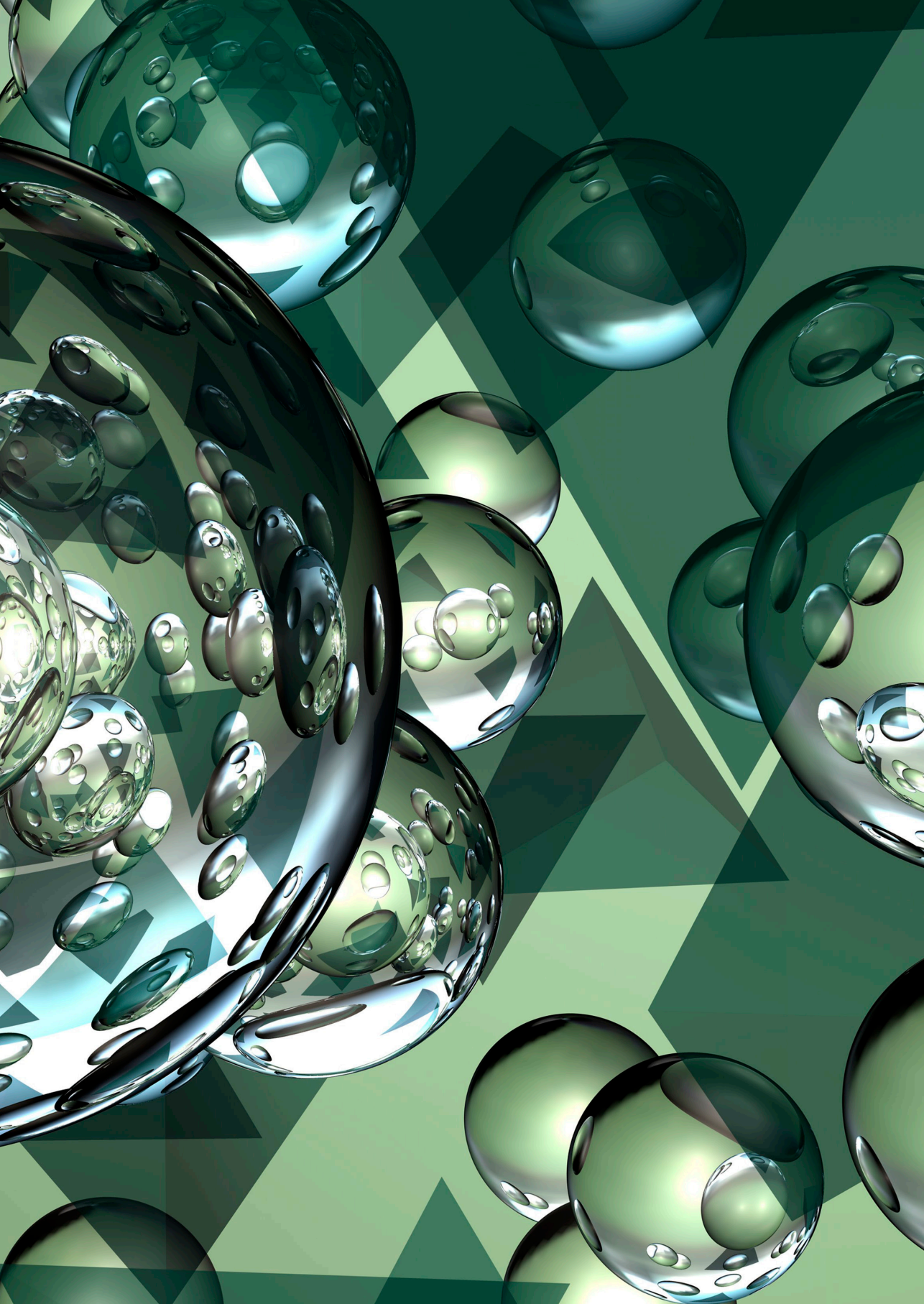
Deel IV: Gevolgen van fosfaatstoornissen

Chronische hypofosfatemie kan leiden tot rachitis met botdeformiteiten bij kinderen en osteomalacie bij kinderen en volwassenen. Hyperfosfatemie kan leiden tot ectopische calcificaties.

De meest voorkomende monogenetische oorzaak van chronische hypofosfatemie is X-gebonden hypofosfatemie (XLH). **Hoofdstuk 9** beschrijft de manifestaties en de complicaties van een cohort van 80 Nederlandse XLH patiënten die geïncludeerd waren in The Observational registry for Rare genetic hypoPHOSphatemia and acquired renal phosphate wasting in the NEtherlanDs (ORPHOS-NED). De meeste patiënten hadden een positieve familieanamnese voor XLH. Patiënten waren kleiner dan gemiddeld en een aantal had overgewicht. De leeftijd ten tijde van de start van XLH-gerelateerde medicatie was negatief gecorreleerd met de lengte ten tijde van inclusie. Bijna 1/3 van de patiënten had gehoorproblemen. Standsafwijkingen van de knie kwamen voor bij 75% van de patiënten en artrose werd gerapporteerd in 51% van de patiënten. Bijna de helft van de patiënten had minimaal 1 orthopedische interventie ondergaan. Nefrocalcinose werd geobjectiveerd in 63.1% van de kinderen en in 33.3% van de volwassenen. De leeftijd ten tijde van start van XLH-gerelateerde medicatie was negatief gecorreleerd met het ontstaan van nephrocalcinose. Deze landelijke studie bevestigt de hoge prevalente van adipositas, vormafwijkingen van het bot, artrose, gehoor verlies en nephrocalcinose in Nederlandse XLH patiënten. Vroeg starten met XLH-gerelateerde behandeling lijkt gunstig te zijn voor de groei maar zou een risicofactor kunnen zijn voor het ontwikkelen van nephrocalcinose.

In **hoofdstuk 10** wordt de associatie tussen de fosfaatconcentratie in serum en calcificaties in de coronair arteriën (CAC) in RS onderzocht. CAC werd gemeten middels elektronenbundeltomografie. Een hoger serum fosfaat was geassocieerd met hogere CAC scores, waarbij het effect in mannen sterker was dan in vrouwen. Exclusie van deelnemers met hyperfosfatemie, chronische nierinsufficiëntie en prevalente cardiovasculaire aandoeningen leidde niet tot een verandering in de resultaten. De resultaten van MR analyses suggereren dat de associatie causaal is, ook bij deelnemers zonder hyperfosfatemie, chronische nierinsufficiëntie en/of cardiovasculaire aandoeningen. Dit suggereert dat hogere serum fosfaat concentraties schadelijk kunnen zijn, zelfs als ze nog binnen de normaalwaarden vallen. Deze bevinding onderstreept het belang van onze eerder benoemde aanbeveling in **Hoofdstuk 2** dat de normaalwaarden voor de fosfaatconcentratie bij mannen en vrouwen herzien moeten worden en waarbij er onderscheid gemaakt moeten worden per geslacht.

In **hoofdstuk 11** worden de resultaten uit dit proefschrift bediscussieerd en de klinische implicaties worden besproken.



Appendices

ABOUT THE AUTHOR

Ariadne Bosman was born on September 29th, 1991 in Wanneperveen.

She graduated *cum laude* at the R.S.G. Stad en Esch in Meppel in 2009 and spent one year traveling in Australia and New Zealand. Thereafter, she studied Medicine at the Utrecht University including a rotation in otolaryngology in Australia, and received her medical degree in November 2016. She started as a resident (ANIOS) in Internal Medicine at the Albert Schweitzer Hospital in Dordrecht in January 2017. In September 2018, she started as a full time PhD-student at the Erasmus MC Bone Center at the Erasmus Medical Center under supervision of Prof. dr. M. C. Zillikens and Dr. B. C. J. van der Eerden. The results of her research are presented in this thesis. She presented her work at several national and international meetings and was awarded a Young Investigator Travel Grant for ASBMR 2022 (Austin, USA). Under the supervision of a steering committee of medical specialists and researchers, she set up ORPHOS-NED, a nationwide observational study of chronic hypophosphatemia, in all academic hospitals in the Netherlands. This thesis presents the result of the first manuscript originating from this nationwide study. She helped to organize meetings for XLH patients, which led to the founding of the Dutch XLH society in 2021. In 2023, she started working as a resident (ANIOS) in Geriatric Medicine at the Medical Center Leeuwarden. In December 2023 she started her training residency in Geriatric Medicine at the department of Internal Medicine at the Medical Center Leeuwarden. She lives together with Nick Bakker and their daughter Anna.

PhD PORTFOLIO

Name PhD student:	Ariadne Bosman
Erasmus MC Department:	Internal Medicine, Erasmus MC Bone Center
PhD period:	September 2018 – May 2024
Promotor:	Prof. dr. M. C. Zillikens
Co-promotor:	Dr. B. C. J. van der Eerden

Conferences: oral presentations	**Year**	**ECTS**
Dutch Society for Calcium and Bone Metabolism meeting, Zeist	2018	0.6
Dutch Endocrine Meeting, Noordwijkerhout	2019	0.6
Dutch Society for Calcium and Bone Metabolism meeting, Athens, Greece	2019	0.9
Internal Medicine Science Days, Sint-Michielsgestel	2020	0.6
Dutch Society for Calcium and Bone Metabolism meeting (online)	2020	0.8
European Calcified Tissue Society, ERA-EDTA CKD-MBD WG Workshop (online)	2021	0.2
European Society for Pediatric Nephrology, Amsterdam	2021	0.9
Dutch Society for Calcium and Bone Metabolism meeting, Zeist	2021	0.8
American Society for Bone and Mineral Research, Austin, USA	2022	1.0
Interdisciplinary Work Group Osteoporosis (IWO), Amersfoort	2022	0.2
Dutch Society for Calcium and Bone Metabolism meeting, Zeist	2022	0.8
Internistendagen, Maastricht	2024	0.8

Conferences: poster presentations	**Year**	**ECTS**
Internal Medicine Science Days, Sint-Michielsgestel	2019	0.6
Dutch Endocrine Meeting, Noordwijkerhout	2020	0.6
European Calcified Tissue Society (online)	2020	0.9
European Calcified Tissue Society (online)	2021	0.9
American Society for Bone and Mineral Research (online)	2021	1.0
European Calcified Tissue Society (online)	2022	1.0
Biomedical Sciences PhD day, Rotterdam	2022	0.3
Internal Medicine Science Days, Sint-Michielsgestel	2022	0.8

Teaching activities	**Year**	**ECTS**
Supervision of 2nd year medical students, systematic review	2018	0.3
Supervision of 2nd year medical students, systematic review	2019	0.3
Lectures on calcium and phosphate metabolism, 2nd year Clinical Technology students	2020	0.5
Supervision of 2nd year medical students, systematic review	2020	0.3

Teaching activities	**Year**	**ECTS**
Lectures on calcium and phosphate metabolism, 2nd year Clinical Technology students	2021	0.5
Lectures on calcium and phosphate metabolism, 2nd year Clinical Technology students	2022	0.5

Courses	**Year**	**ECTS**
Microsoft Excel 2010 (Basic)	2018	0.3
Microsoft Access 2010 (Basic)	2018	0.3
Microsoft Access 2010 (Advanced)	2018	0.4
Systematic literature retrieval in Pubmed	2018	0.4
Genetics for Dummies	2018	0.6
Basic Introduction Course on SPSS	2018	1.0
Survival Analysis	2018	0.6
Endnote	2018	0.2
Systematic literature retrieval in Embase	2019	0.2
Reviews: project management, other databases and Endnote	2019	0.2
CPO-course: Patient Oriented Research	2019	0.3
OpenClinica	2019	0.3
Basic Course on 'R'	2019	2.0
GWAS Blitz course	2019	0.9
Scientific Integrity	2019	0.3
Follow-up Photoshop and Illustrator CC	2019	0.3
SNP Course: SNPs and Human Diseases	2019	2.0
Indesign CC	2019	0.2
BROK® (Basic course Rules and Organisation for Clinical researchers)	2020	1.5
Biomedical English Writing	2020	2.0
GE03 Advances in Genome-Wide Association Studies	2021	1.4
Castor	2022	0.2

Other	**Year**	**ECTS**
Regionale Endocrinologie Bespreking Rotterdam, Rotterdam	2019	0.2
Fractuurpreventie Spreekuur, Rotterdam	2019	0.2
4th International Mendelian Randomization Conference, Bristol, UK	2019	1.6
Quality of Life for Osteogenesis Imperfecta congress, Amsterdam	2019	0.6
Workshop on How to Design and Pitch a Good Poster	2020	0.2
UK Biobank Scientific Conference	2020	0.2
American Society for Bone and Mineral Research (online)	2020	0.5
European Calcified Tissue Society webinars	2020	0.2
Dutch Society of Endocrinology – information folder on XLH	2020	0.2

Other	**Year**	**ECTS**
Organizing information event for patients with hypophosphatemia (online)	2020	0.5
European Calcified Tissue Society webinar Rare Bone Diseases, co-chair	2021	0.2
Expertise Connected (VSOP)- Development of a website on XLH	2021	0.4
Organizing information event for XLH patients, Rotterdam	2022	0.5

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Campos-Obando N*, **Bosman A*,** Kavousi M, Medina-Gomez C, van der Eerden BCJ, Bos D, Franco OH, Uitterlinden AG, Zillikens MC. Genetic Evidence for a Causal Role of Serum Phosphate in Coronary Artery Calcification: The Rotterdam Study. J Am Heart Assoc. 2022 Aug 2;11(15):e023024.

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Bosman A, Campos-Obando N, de Keyser CE, Stricker BH, Zillikens MC. Diuretic use and serum phosphate: Rotterdam Study and UK Biobank. Accepted for publication in J Endocr Soc

*Shared first author

Submitted

Bosman A, Campos-Obando N, Ramakers C, Zillikens MC. Serum phosphate in the general population: a need for sex-specific reference intervals. Submitted

Publication not in this thesis

Bosman A, Hobbel HK, Lushchyk T, Kuitwaard K, van Bommel EFH. Progressive visual decline in a Rotterdam harbor crane operator. Neth J Med. 2018 Dec;76(10):452.

Van Velsen EFS, Geeraedts TEA, **Bosman A**, Zillikens MC. Thermal Ablation for Treating Tumor-induced Osteomalacia in a Patient With IV Phosphate Dependency. JCEM Case Rep. 2023 Jul 27;1(4):luad086.