The fate of phosphate in diabetes
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Chapter 1

Introduction and aims

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Diabetes is one of the leading non-communicable diseases worldwide, with a prevalence that has risen from 108 million individuals in 1990 to 537 million individuals in 2021, representing approximately 10% of the global population. (1) Diabetes is a heterogeneous disease, characterized by elevated blood glucose levels resulting from either absolute insulin deficiency (in type 1 diabetes) or reduced peripheral insulin sensitivity (in type 2 diabetes). Individuals with diabetes face a risk of developing both acute and chronic complications. In the short term, an acute hyperglycemic crisis such as diabetic ketoacidosis (DKA), often seen in type 1 diabetes, is a life-threatening condition with a mortality rate of more than 5%. (2) In the long term, microvascular complications such as retinopathy, neuropathy, and nephropathy, as well as macrovascular complications such as peripheral vascular disease, stroke, and coronary artery disease, are commonly observed. (3) These complications contribute to the overall burden of the disease and its associated morbidity and mortality. The global mortality rate attributed to vascular complications in diabetes has risen by 37.9% over the past two decades. (4) To address these challenges, it is crucial to identify new pathways that contribute to the development of diabetes, as well as its complications. Metabolic deregulations are frequently observed in diabetes and can involve alterations in lipid, amino acid and energy metabolism, as well as dysregulation of insulin signaling and oxidative stress pathways. (5) Deregulated phosphate metabolism may contribute to both the onset of diabetes and its cardiovascular complications.

**Phosphate homeostasis**

Phosphate plays a crucial role in various biological processes. (6,7) It serves as a building block of phospholipid membranes that make up cell structures, is involved in the production of adenosine triphosphate (ATP) for energy metabolism, and acts as a buffer for maintaining urinary pH. (8) The vast majority of phosphate in the body, approximately 85%, is stored in bone as calcium phosphate. The remaining 15% is divided between the extrasosseous intracellular (14%) and extracellular (1%) compartments. In the extracellular fluid, phosphate can be bound to different minerals, including calcium. Phosphate homeostasis is tightly regulated. The kidneys are key regulators of phosphate homeostasis. (9,10) Specifically, sodium-phosphate co-transporters (NaPi2a/c) residing in the proximal tubular epithelium of the kidney orchestrate phosphate reabsorption under the control of hormones, including parathyroid hormone and fibroblast growth factor 23 (FGF23). A schematic overview of phosphate metabolism including main phosphate-regulating hormones is shown in Figure 1.

Phosphate is an essential mineral, implying that dietary sources are essential to meet the body’s requirements. (6) However, dietary phosphorus is plentiful in the
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Western diet. Protein-rich food products such as meat, fish, nuts, and dairy products contain organic phosphate. Inorganic phosphate is present in many food additives. Therefore, highly processed foods, including fast food, contain high amounts of inorganic phosphate. (11,12) Inorganic phosphate is efficiently absorbed in the intestine, making processed foods important sources of highly bioavailable phosphate. (13–15) Intestinal phosphate uptake occurs via active transport by sodium–phosphate cotransporters (NaPi–IIb), and is positively regulated by active vitamin D. Additionally, passive phosphate transport takes place through a paracellular pathway, which is diffusion-driven and mostly determined by dietary phosphate intake.

Figure 1. The central role of FGF23 in the regulation of phosphate balance.

FGF23 is released from bone cells in response to increased dietary phosphate intake or decreased phosphate excretion in the glomerulus (caused by a low estimated glomerular filtration rate, or eGFR). FGF23 inhibits phosphate reabsorption via NaPi2a transporter. As a result, renal phosphate clearance increases, restoring phosphate balance. Both parathyroid hormone (PTH) and active vitamin D enhance the secretion of FGF23 and, in a negative feedback loop, are inhibited by FGF23. Furthermore, vitamin D can also enhance phosphate absorption in the intestines by inducing the expression of NaPi2b, contributing to phosphate loading and the secretion of FGF23. Vitamin D and PTH also play a role in regulating the levels of calcium and phosphate, and these minerals directly affect the hormonal pathways involved. Hyperphosphatemia and hypocalcemia stimulate the release of PTH. Hypocalcemia also directly activates vitamin D, while hyperphosphatemia inhibits this activation.

Chapter 1

Phosphate and glucose metabolism

Individuals that suffer from DKA develop acute deregulations in phosphate metabolism. (16) DKA is characterized by the presence of hyperglycemia and metabolic acidosis, which results from insulin deficiency with consequently unopposed lipolysis and oxidation of free fatty acids, resulting in ketone body production. (17) Large shifts in phosphate between the intra- and extracellular compartments appear during DKA (Figure 2).

Initially, due to hypertonicity resulting from hyperglycemia, phosphate shifts to the extracellular compartment. (18) Moreover, there is a reduction in the cellular uptake of phosphate from the extracellular compartment. Also, acidosis reduces glycolysis by the enzyme 6-phosphofructo-1-kinase and the intracellular uptake of phosphate. (19–21) As a result of subsequent hyperphosphatemia, large amounts of phosphate are being filtered into the pre-urine, causing osmotic diuresis with little reabsorption of the filtered phosphate. This reduced reabsorption can be explained by acidosis, that has a direct effect on the brush borders of the proximal tubule, where transepithelial phosphate transport is partly regulated by pH levels. (22,23) This massive phosphaturia seems to serve a purpose, since phosphate is used as an important urine buffer during metabolic acidosis.

Simultaneously, both insulin deficiency and glycosuria inhibit renal phosphate reabsorption, further contributing to phosphaturia and net phosphate loss. (24) At initial presentation, individuals with DKA often display normophosphatemia or even hyperphosphatemia, while hypophosphatemia is rare. Apparently, the shift of phosphate from the intra- to extracellular compartment often exceeds the net urinary loss of phosphate, explaining the initial low prevalence of hypophosphatemia. However, during fluid and insulin administration as part of the treatment of DKA, phosphate shifts back to the intracellular compartment, leading to hypophosphatemia. Literature on (time of) nadir phosphate levels in DKA, the magnitude, determinants and consequences of hypophosphatemia in DKA is scarce or even lacking. (25) Furthermore, the effects of glucose loading on phosphate metabolism, including FGF23 secretion, are poorly understood.

In addition to the aforementioned acute changes in phosphate metabolism in the acute hyperglycemic setting, persons with diabetes may develop chronic deregulations in phosphate and FGF23 homeostasis. Individuals with diabetic nephropathy often present with deregulated bone and mineral metabolism, which is one of the hallmarks of chronic kidney disease (CKD) in general. In parallel with declining kidney function, individuals with diabetic nephropathy develop hyperparathyroidism, reflected by increased PTH levels, which is a compensatory response to higher phosphate and lower calcium and vitamin D levels, as caused by renal
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failure. Prior to the increase in phosphate and PTH levels, the production of FGF23 levels in osteocytes is stimulated. FGF23 levels can increase more than 1000-fold in kidney failure, potentially with glyceryl-3-phosphate as renal trigger. (26–28) FGF23 levels tend to normalize in most patients after successful kidney transplantation, although levels remain elevated in some patients. (29) In addition to impaired kidney function in CKD, diabetes in itself also seems to influence phosphate and FGF23 levels. (30)

Several studies found a link between insulin resistance and elevated FGF23 levels. Preclinical studies showed that insulin is a negative regulator of FGF23, by activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling transcription factor forkhead box protein O1 (FOXO1). (31)

In an elegant set of experiments, it was shown that insulin treatment in cultured cells and mice inhibited FGF23 gene expression. (31) Furthermore, elevated FGF23 levels were found in insulin-deficient mice, which could be normalized by administration of exogenous insulin. Therefore, insulin might be able to directly decrease FGF23, independent of inflammation or changes in kidney function. Vice versa, FGF23−/− mice are hypoglycemic and have increased peripheral insulin sensitivity. (12) Furthermore, mice with a PHEX mutation, leading to FGF23 overexpression, presented with hyperglycemia and hypoinsulinemia. (32) Also, obesity and impaired glucose metabolism seems to be present in human counterparts

Figure 2. Schematic overview illustrating the hypothesized course of plasma inorganic phosphate in DKA, and potential factors driving changes in plasma phosphate during DKA.
with X-linked hypophosphatemic rickets, although increased diabetes risk has not been reported. (33)

**Consequences of deregulated phosphate metabolism in diabetes**

Many factors may contribute to the deregulations in phosphate and FGF23 homeostasis in diabetes. The second part of this thesis will elaborate on the consequences of deregulated phosphate and FGF23 homeostasis in diabetes. Strong associations between phosphate and FGF23 levels and cardiovascular and all-cause mortality have been reported in many studies in populations ranging from healthy individuals to individuals with kidney failure. (34–37) Notably, several studies have shown that FGF23 and phosphate are associated with major adverse cardiac events and premature mortality in individuals with type 2 diabetes with preserved or only mildly impaired kidney function. (30,34,36,38–40)

Although some data implicate FGF23 also in vascular calcification(41), other studies do not support a direct link. (42,43) Alternatively, it could be hypothesized that FGF23 contributes to adverse outcomes through mechanisms other than vascular calcification. Preclinical studies point towards offtarget effects of FGF23 on cardiomyocytes through FGF receptor 4 (FGFR4), inducing left ventricular hypertrophy. (44,45) A higher FGF23 level has been linked with volume retention, through upregulation of the sodium-chloride cotransporter which results in increased sodium reabsorption in the distal tubule. This deleterious pathway could drive the relationship between FGF23 and adverse long-term outcomes, including mortality. (46,47)

Thus, although FGF23 may contribute to adverse outcomes through pathways other than vascular calcification, higher phosphate levels have been much more consistently linked with vascular calcification (Figure 3). Calcification of the vascular wall, specifically the tunica media layer, is a hallmark of advanced diabetes and a strong, independent risk factor for CVD. (48) Deregulations in mineral metabolism, and calcium and phosphate homeostasis in particular, set the stage for accelerated vascular calcification. This paradigm has been more extensively studied in individuals with CKD, where deregulation of phosphate and calcium metabolism parallel progressive loss of kidney function. (49) High-normal plasma phosphate concentrations have unfavorable effects on cardiovascular health by stimulating osteochondric differentiation of vascular smooth muscle cells (VSMCs), leading to media calcification present in CVD. On one hand, high phosphate levels induce VSMC remodeling, mediated by phosphate-transporters I and II (PiT-1 and PiT-2, respectively). (50,51) This triggers VSMC osteochondric differentiation as well as matrix mineralization, leading to vascular wall stiffening. An increasing number of publications suggest that the presence of hyperglycemia accelerates plasma
Figure 3. Hyperphosphatemia drives key processes that promote vascular calcification in diabetes.

Phosphate-induced osteochondric differentiation of VSMCs. (52–54) At the same time, high phosphate levels promote the conversion of primary into secondary calciprotein particles (CPPs), which in turn promote oxidative stress and inflammation. (55,56) Primary CPPs contain amorphous calcium phosphate, while secondary CPPs contain crystalline calcium phosphate. (57–59) The ability of secondary CPPs to induce calcification, particularly in VSMCs, indicates that the rate of primary-to-secondary CPP transition is an indicator of the serum’s anticalcifying buffer capacity. Increased formation and maturation, as well as defective clearance of CPP, is an important novel risk factor for cardiovascular disease. (60,61) This is further supported by the fact that amorphous primary CPPs have a minor effect on macrophage cells, while secondary CPPs induce oxidative stress and inflammation in macrophages, and oxidative stress, inflammation, and calcification in human aortic smooth muscle cells. (62–64) To quantify the serum’s anticalcifying buffer capacity, the serum $T_{50}$ calcification propensity test has been developed.
This test measures how quickly primary CPPs are transformed to secondary CPPs in a patient’s blood sample, with lower serum $T_{50}$ values indicating a higher propensity for calcification. A low serum $T_{50}$ value is an independent predictor of cardiovascular morbidity and mortality in various populations, including the general population, kidney transplant recipients and individuals with CKD. Individuals with diabetes are thought to be extra vulnerable to the development of phosphate-induced vascular calcifications, compared to those without diabetes, even with phosphate levels are in the normal range. However, the relationship between serum $T_{50}$ and indices of diabetes, such as glycated hemoglobin (HbA1c) and diabetes therapy, clinical outcomes has not been addressed in a diabetes population.

**Objectives and Outline of Thesis**

The general objective of this thesis is to study the interrelationship between phosphate and glucose metabolism and assess the clinical consequences of deregulated phosphate homeostasis in individuals with (susceptibility to) diabetes.

In Chapter 2 we examined the incidence, determinants, and clinical implications of hypophosphatemia in DKA. We evaluated the time to nadir phosphate levels and analyzed whether hypophosphatemia in DKA was associated with prolonged hospitalization, morbidity, or mortality. Chapter 3 focuses on investigating the cross-talk between FGF23 and glucose homeostasis. We assessed the effect of glucose loading on changes in plasma phosphate and FGF23 and investigated the association of FGF23 with incident diabetes. Subsequently, in Chapter 4, we studied the predictive value of FGF23 on the development of post-transplant diabetes in kidney transplant recipients.

Next, we conducted studies that investigated consequences of deregulated phosphate metabolism in individuals (susceptible) to diabetes. In Chapter 5, we assessed whether the association between plasma phosphate and mortality in the general population was modified by diabetes status. Then, in Chapter 6, we studied serum calcification propensity in type 2 diabetes. We assessed i) the determinants of serum $T_{50}$, including plasma phosphate, and ii) the association with cardiovascular mortality and all-cause mortality. In Chapter 7, we subsequently studied whether HbA1c was associated with calcification propensity. In Chapter 8, we studied whether the association between FGF23 and mortality was modified by volume status in the general population. Lastly, in Chapter 9, we investigated the effects of high versus low dairy intake on markers of bone and joint health and phosphate metabolism in individuals susceptible to diabetes.

Chapter 10 provides the general discussion of this thesis and future perspectives.
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