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Chapter 10
General discussion and future perspectives
Diabetes could be considered a pandemic disease, given its rapidly increasing global prevalence over recent decades. It is expected that the global number of individuals affected will rise to 642 million by 2040. (1) In the Netherlands alone, there are approximately 1.1 million individuals (6.5% of the population) living with diabetes, resulting in a significant burden on both individuals and society, as continuous monitoring and management are necessary. (2) Additionally, diabetes-related vascular complications are of major concern, adding to the already substantial health and economic burden of the disease. Despite extensive research into risk factors and interventions in diabetes, efforts to reduce the rise in diabetes-related morbidity and mortality have been mostly unsuccessful. (3) Therefore, strong efforts are needed to identify modifiable pathways that play a role in the development of diabetes and related complications. Phosphate metabolism emerges as a pathway deserving consideration.

The aim of this thesis was to assess the role of phosphate metabolism in diabetes and diabetes related outcomes. Firstly, we conducted studies that assessed the relationships between phosphate and glucose metabolism. Secondly, we explored the impact of phosphate metabolism on diabetes related outcomes.

Measuring plasma phosphate during diabetic ketoacidosis – a sacred cow?
In Chapter 2 we retrospectively investigated the prevalence of phosphate deregulations during diabetic ketoacidosis (DKA). We observed that both hyper- and hypophosphatemia are highly prevalent during DKA (66% and 74%, respectively). Transcellular shifts of phosphate from the intra- to the extracellular compartment are a result of hyperglycemia and acidosis, thereby promoting osmotic diuresis. (4) Although osmotic diuresis, acidosis and insulin deficiency also stimulate phosphaturia, the net phosphate loss is often exceeded by the transcellular phosphate shifts. (5,6) Therefore, patients with DKA frequently present with initial hyperphosphatemia.

During fluid and insulin administration, as treatment for DKA, phosphate rapidly shifts back to the intracellular compartment. (4) Insulin facilitates cellular phosphate uptake by sodium-dependent transporters, thereby stimulating the intracellular formation of glucose-6-phosphate which is the first intermediate for glycolysis. (7) Our results suggest that the degree of acidosis upon presentation is the most prominent determinant of hypophosphatemia and that the time to nadir of phosphate levels is around 16 hours after admission. It is worth noting that plasma phosphate represents only 1% of the body’s total phosphate stores and does not reflect the overall body reservoir. (8)

In approximately one-third of the cases, we observed a lack of adherence to the Dutch guideline, which states that plasma phosphate should be measured
1-3 times during the treatment of DKA and that intravenous phosphate should be administered if levels fall below 0.30 mmol/L. (9) Furthermore, in practice, phosphate levels were often not reassessed after diagnosing hypo- or hyperphosphatemia, irrespective of whether treatment was initiated. Notably, individuals with untreated hypophosphatemia did not experience adverse outcomes, such as respiratory or cardiac failure, or prolonged hospital stays. However, the observational and retrospective design and the small sample size of the study limits firm conclusions.

There is an ongoing debate regarding the necessity of intervening with phosphate replacement therapy in case of hypophosphatemia. The primary peril associated with hypophosphatemia is a potential depletion of erythrocyte 2,3-diphosphoglycerate (2,3-DPG), which has led to current recommendations for including phosphate supplementation in the regimen. (10) However the occurrence of adverse events as a result of hypophosphatemia, such as the occurrence of cardiac and respiratory failure, is limited to a few case reports. (11,12)

Based on the findings in Chapter 2, we argue that the Dutch guideline needs to be reevaluated for several reasons. Firstly, phosphate levels exhibit significant fluctuations within a relatively short period of time, rendering phosphate measurement at initial presentation uninformative.

Hypophosphatemia is rarely observed at that stage, and phosphate levels at that point cannot predict the occurrence or severity of subsequent hypophosphatemia. Hypophosphatemia is more likely to manifest during the recovery phase from acidosis, which is about 11-24 hours after presentation and often not the moment when physicians typically assess plasma phosphate levels. (13,14) Secondly, even in the presence of hypophosphatemia, the usefulness of phosphate measurement and treatment remains questionable, as studies have failed to demonstrate any benefit of phosphate replacement therapy in DKA and have not shown harmful consequences associated with hypophosphatemia. (15–18) It is possible that the observed hypophosphatemia during fluid resuscitation and insulin treatment in DKA is a response to the intervention, rather than a pathophysiological phenomenon. Phosphate supplementation may even hinder the body’s natural efforts to restore phosphate balance, resulting in subsequent hyperphosphatemia. Lastly, excessive phosphate treatment can lead to severe hypocalcemia and hypomagnesemia, as a result of transient hypoparathyroidism. (19,20)

Phosphate metabolism in response to alterations in glucose metabolism
Gaining understanding of phosphate levels in DKA not only sheds light on the complex nature of phosphate metabolism in response to acute glycemic disturbances, but also paves the way for a deeper exploration of the potential mechanisms that
explain phosphate metabolism in response to alterations in glucose metabolism. Various studies have shown that insulin and glucose loading induce alterations in plasma phosphate levels. (6,17,21,22) In a stable, out-hospital setting, plasma phosphate levels appear to be similar in individuals with and without type 2 diabetes (T2D) (as observed in Chapter 5). However, in the context of acute hyperglycemia or insulin therapy, lower phosphate levels are reported. (23–25) Although insulin stimulates renal phosphate reabsorption(6), this effect might be outweighed by the impact of insulin on cellular phosphate uptake. (7)

According to literature, levels of the main phosphate-regulating hormone fibroblast growth factor 23 (FGF23) are also affected by glucose metabolism, specifically leading to lower FGF23 levels. (22,26,27) Consequently, we sought to explore whether the observed effects of glucose loading on FGF23 were phosphate-independent. In Chapter 3 we assessed the effect of glucose loading on FGF23 and its temporal relationship with changes in plasma phosphate in a post-hoc analysis of a cohort that underwent an oral glucose tolerance test. Here, we found that changes in plasma phosphate were preceded by changes in FGF23, implying a phosphate-independent effect of glucose loading on FGF23. The underlying factors driving this decrease in FGF23 secretion remain a subject of inquiry. Experimental studies have demonstrated that insulin (growth factors) down-regulate FGF23 production in osteocytes by inhibiting the transcription factor forkhead box protein O1 (FOXO1) through the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling pathway. (27) Additionally, this insulin-mediated effect stimulates the expression of Phosphate-regulating gene homologous to endopeptidase on X chromosome (PHEX), resulting in increased expression of FGF23. (28)

Glucose metabolism in response to alterations in phosphate metabolism

While glucose metabolism affects plasma phosphate levels, there is limited literature available on the reciprocal relationship between plasma phosphate and glucose metabolism. Studies conducted in rats and dogs have shown that phosphate depletion leads to glucose intolerance and reduced insulin secretion. (29,30) However, direct evidence for the impact of phosphate depletion on pancreatic insulin secretion by pancreatic islets is currently lacking, and the precise mechanism through which phosphate depletion may affect insulin secretion remains unknown, although an important role for ATP has been suggested. Interestingly, a large study involving over 71,000 women demonstrated an association between higher dietary phosphate intake and an increased risk of developing T2D. (31) This effect could be mediated by FGF23, as phosphate-rich diets have been shown to
increase circulatory levels of FGF23. (25,26) FGF23 itself has been linked to different measures of insulin resistance (34), including HOMA-IR (35) and resistin. (36)

Considering the proposed role of FGF23 in insulin resistance, we conducted a prospective study to investigate the association between FGF23 and future diabetes in kidney transplant recipients (KTR).

KTR are at high risk to develop post-transplant diabetes (PTDM) with a prevalence up to 50%. (37,38) Furthermore, KTR experience pathologically elevated levels of FGF23. Although there may be a reduction in these levels following transplantation, overall FGF23 levels remain elevated compared to those observed in the general population. (39) In the study presented in Chapter 4, we found that KTR in the highest tertile of FGF23 are at increased risk for the development of PTDM, independent of diabetes-related or transplant-related risk factors.

Having established an association between FGF23 and the occurrence of diabetes in a population with pathologically elevated FGF23 levels, we were intrigued by the possibility of extrapolating these findings to the general population. In Chapter 3, we conducted a prospective cohort study among individuals from the general population. We observed a similar association between FGF23 and incident T2D in this cohort, whereby the impact of FGF23 on obesity partially contributed to this association.

The potential mechanism underlying the association between FGF23 and reduced insulin sensitivity and diabetes remain elusive. Nonspecific binding of FGF23 may mediate off-target effects in tissues and organs not previously considered as targets. The PLCγ/calcineurin (CN)/nuclear factor of activated T cells (NFAT) signaling pathway can be activated by FGF23 binding, independent of αKlotho. The PLCγ/CN/NFAT pathway plays a critical role in regulating β-cell growth, function, and insulin synthesis and secretion. (40) Inhibition of this pathway causes hyperglycemia and decreased insulin levels, whereas sustained activation can detrimentally affect β-cell proliferation and function. Furthermore, mice lacking αKlotho display pancreatic islet atrophy and reduced insulin levels, while overexpression of αKlotho enhances insulin secretion and intracellular calcium response. (41,42) Thus, coexistence of conditions marked by supraphysiological levels of FGF23 and diminished expression of its coreceptor αKlotho may potentially instigate a decline in insulin sensitivity. Studies in mice with PHEX mutations leading to FGF23 overexpression have demonstrated concurrent hyperglycemia and hypoinsulinemia. (43,44) Interestingly, individuals with X-linked hypophosphatemia (XLH), a rare disease caused by this PHEX mutation and characterized by renal phosphate wasting and hypophosphatemia, were also more prone to develop obesity, while insulin sensitivity in persons with XLH needs to be further evaluated. (45,46) This parallels the results observed in Chapter 3, where the
association between FGF23 and diabetes was largely driven by obesity. Plausible adaptive mechanisms may exist that might prevent the premature onset of diabetes in patients with XLH.

In conclusion, we can state that there are bidirectional connections between insulin and phosphate metabolism. Figure 1 provides a schematic overview of the proposed mechanisms involving insulin and phosphate homeostasis.

Future studies to further elucidate these bidirectional pathways are of interest. To assess the role of phosphate metabolism in responses to (alterations in) glucose metabolism, we are currently conducting a prospective study on the temporal relationship of phosphate and FGF23 during DKA. It would also be of interest to investigate the same phenomena during a euglycemic hyperinsulinemic clamp. Additionally, further investigating glucose homeostasis in animal models such as klotho knockout mice or FGF23 overexpression models could elucidate the underlying mechanisms and pathways involved.

**Potential mechanisms connecting glucose and phosphate deregulations with vascular calcification**

All of the aforementioned studies point towards interaction between deregulated phosphate metabolism and the pathophysiology of diabetes. In the second part of the thesis, we focused on exploring the relationship between phosphate metabolism and the consequences of diabetes.

Deregulations in mineral metabolism, particularly in phosphate homeostasis, can contribute to accelerated development of vascular calcification. Elevated plasma phosphate levels have detrimental effects on cardiovascular health by promoting the remodeling of vascular smooth muscle cells (VSMCs) through the action of phosphate-transporters I and II (PiT-1 and PiT-2). (47,48) This remodeling process induces VSMC osteochondrial differentiation and matrix mineralization, resulting in stiffening of the vascular wall. Additionally, elevated plasma phosphate levels facilitate the conversion of primary calciprotein particles (CPPs) into secondary CPPs, reflecting increased calcification propensity. (49,50) Emerging evidence suggests that hyperglycemia accelerates plasma phosphate-induced osteochondric differentiation of VSMCs, potentially through the upregulation of PiT-1 phosphate transporters. (51–54) Consequently, individuals with T2D are thought to be particularly susceptible to the development of phosphate-induced vascular calcification, even with plasma phosphate levels within the normal range. In Chapter 5, we showed the findings of a large prospective population-based study cohort. We found that the association between elevated plasma phosphate levels, even within the normal range, and all-cause mortality is stronger in individuals with T2D, compared to those free from diabetes at baseline.
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We further investigated this higher susceptibility to phosphate in individuals with T2D by assessing calcification propensity and glycemic control (HbA1c). As described in Chapter 6, our findings in a prospective cohort study among individuals with T2D, treated in primary care, indicated that higher levels of HbA1c were a significant determinant of lower $T_{50}$ time, which reflects stronger calcification propensity. Persons with higher HbA1c levels or higher systolic blood pressure, those who use insulin, and individuals with a longer history of T2D were found to have shorter $T_{50}$ time.

Expanding upon these observations, we found that a lower serum $T_{50}$ time was associated with cardiovascular mortality in another cohort of individuals with T2D, treated in secondary care (Chapter 7). A similar relationship was observed for all-cause mortality, but not for non-CV mortality, which strengthens the observation that serum $T_{50}$ is specifically important for CV-related morbidity and mortality. Furthermore, plasma phosphate was the most important determinant of serum $T_{50}$.

Figure 1. Mechanisms proposed that link glucose and phosphate metabolism.

FGF23 is a phosphaturic hormone that inhibits renal phosphate reabsorption and decreases plasma phosphate. A decrease in plasma phosphate might potentially induce insulin resistance via reduced ATP levels in β-cells (although direct evidence is lacking). FGF23 itself is believed to promote insulin resistance by sustained activation of the PLCγ/CN/NFAT pathway, which can influence β-cell proliferation and function. Furthermore, FGF23 may contribute to insulin resistance by promoting adiposity, a known risk factor for insulin resistance. Conversely, insulin (and growth factors) can down-regulate FGF23 production in osteocytes by inhibiting the transcription factor forkhead box protein O1 (FOXO1) through the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling pathway.
The role of plasma phosphate in vascular calcification is even more pronounced in individuals with diabetic nephropathy (20-40% of all individuals with T2D). (55) Hyperphosphatemia is present in more than 50% of the individuals with chronic kidney disease (CKD) stage 3-5, and the morbidity and mortality as a result of vascular calcification is excessive among this population, especially in those with concomitant T2D. (56,57) Both T2D and CKD seem to trigger deregulations in phosphate and FGF23 metabolism, culminating in a high risk of such deregulations in diabetic nephropathy.

Hence, there is a significant opportunity to exert a beneficial influence on cardiovascular health and even reduce the incidence of cardiovascular morbidity and mortality in individuals with T2D, in particular those with diabetic nephropathy, by manipulating phosphate concentrations. Most studies have primarily been of experimental or observational nature, leaving a gap that needs to be addressed to translate the promising findings from these studies into strategies that can effectively reduce phosphate levels in T2D. Clinical trials aimed at modulating phosphate levels, such as through the administration of phosphate supplementation or phosphate-lowering therapies, may offer a promising avenue for exploring the impact of altered phosphate homeostasis on calcification propensity in individuals with T2D.

**FGF23, volume status and clinical outcomes**

In addition to phosphate-related adverse outcomes, we aimed to elucidate the role of FGF23 in cardiovascular morbidity and mortality. The mechanism by which FGF23 contributes to cardiovascular disease appears to be driven by vascular calcification only to a small extent. Rather, factors such as volume overload and left ventricular hypertrophy are suggested to be key players. (58–61) FGF23 has exhibited robust associations with multiple indices of volume overload, including MR-proANP and NTproBNP, which are both established as reliable markers for assessing left ventricular wall strain. (62) Higher levels of FGF23 have been linked to the development of new-onset heart failure, as well as indicating disease progression and prognosis within the heart failure population. (63–65) Additionally, FGF23 has been associated with left ventricular hypertrophy (66), which is a long-term consequence of volume overload.

To explore whether the association between FGF23 and mortality was driven by changes in volume status, we conducted a mediation study among individuals with T2D, as presented in Chapter 8. We observed that volume status, as measured by NT-proBNP, is a significant mediator in the association between FGF23 and all-cause mortality.
The pathophysiologic mechanisms underlying the association between FGF23 and volume overload might be in the direct action of FGF23 on NaCl cotransporters (NCC) in the renal distal tubule. (60) This regulation occurs through a signaling mechanism involving the FGF receptor/αKlotho complex, extracellular signal-regulated kinase 1/2 (ERK1/2), serum/glucocorticoid-regulated kinase 1 (SGK1), and with-no lysine kinase-4 (WNK4). Mouse models with deficiencies in FGF23 or αKlotho exhibited reduced renal sodium reabsorption and decreased membrane expression of NCC in distal tubules. Conversely, when wild-type mice were injected with recombinant FGF23 or when they had elevated levels of endogenous FGF23, distal tubular sodium uptake and NCC membrane abundance increased. This led to volume expansion, hypertension, and cardiac hypertrophy, with these effects being dependent on αKlotho and dietary sodium levels.

**Interventions that reduce plasma phosphate and FGF23 levels**

Given the detrimental associations between deregulated phosphate and FGF23 on the one hand, and an increased cardiovascular morbidity and mortality risk on the other hand, it seems important to explore potential interventions to target deregulations in phosphate metabolism. As outlined above, the exact impact of diabetes on phosphate homeostasis remains unclear, since phosphate levels may be normal or low in uncomplicated diabetes, whereas hyperphosphatemia may develop in diabetic nephropathy. Circulating FGF23 starts to rise early in the course of diabetic nephropathy, before the development of other abnormalities, such as elevated PTH, lowered calcium, elevated phosphate or lowered vitamin D. (67) Lowering FGF23 and/or phosphate levels may therefore be a valuable therapeutic target, particularly in populations with diabetic nephropathy.

Dietary interventions may be an important non-pharmacological strategy to reduce FGF23 and phosphate levels. Although strong associations between phosphorus intake and plasma phosphate levels were reported in cross-sectional studies (68,69), subsequent studies involving dietary interventions, with both high and low phosphorus content, did not find significant changes in plasma phosphate levels. (70,71) Notably, alterations in plasma FGF23 have been observed both after shortterm (a few days/weeks) (71,72) and after long-term (up to a year) (73), with the exception of one study that reported altered FGF23 levels after 4 weeks but not after 8 weeks. (70) Given that the studies conducted thus far predominantly utilized a dietary phosphate supplement, our objective was to explore a dietary intervention characterized by a naturally high phosphate content. Chapter 9 delved in the consequences of high vs low dairy intake, given the high phosphorus content of dairy, in individuals susceptible to T2D. The primary objective of the study was to evaluate markers of bone and joint metabolism after both diets, which exhibited
significant improvement following high dairy intake. Remarkably, we observed no structural changes in plasma phosphate levels following both diets. However, high dairy intake resulted in an increase in urinary phosphate excretion and FGF23 displayed a trend towards an increase.

Our findings are in line with previous work showing that high dietary protein intake, which can lead to an increased dietary load of both phosphate and acid, has been associated with higher FGF23 levels. Conversely, a very low-protein diet (0.3g/kg BW/day) can decrease FGF23 levels. (74) However, achieving and maintaining overall protein reduction in practice may prove challenging, and may induce catabolism with potential adverse health outcomes. Another trial investigated the impact of consuming plant-based protein instead of animal-based protein, as plant-based protein is typically accompanied by lower bioavailable phosphorus. This dietary intervention resulted in lower FGF23 levels. (75) An alternative approach focuses on correcting magnesium deficiency. Magnesium deficiency has been found to induce elevated FGF23 levels in rats, and magnesium intake has been inversely correlated with FGF23 levels in humans. (76–78) These findings suggest that correcting magnesium deficiency may lower FGF23 levels. However, in a clinical trial, oral magnesium supplementation did not lead to lower FGF23 levels. (79) Additionally, energy deprivation or low caloric intake has been linked to lower FGF23 levels, although prospective studies are needed to confirm this observation. (80)

Phosphate binders reduce intestinal phosphate absorption and may effectively lower plasma phosphate. Various trials investigated the effect of phosphate binders on plasma phosphate and FGF23 levels in persons with CKD. (81–83) Mainly non calcium-based polymers, such as sevelamer and lanthanum can significantly decrease FGF23 levels. One study among individuals with diabetic nephropathy showed that sevelamer can reduce both FGF23 and HbA1c. (84) This effect could be attributed to the binding and removal of dietary advanced glycated end products, which contribute to inflammation and oxidative stress. On the contrary, calcium-based phosphate binders in several studies did not reduce FGF23 or even led to higher FGF23 levels. (81,85) This is most likely due to the calcium component which could influence FGF23, offsetting the effects of calcium-based phosphate binders. Another drug that influences gastrointestinal absorption of phosphate is tenapanor, a nonabsorbable oral inhibitor of sodium-hydrogen exchanger 3 (NHE3). A study in dialysis patients showed that tenapanor can induce a decrease in serum phosphate as well as FGF23 levels. (86) Subsequently, the AMPLIFY trial showed that in hemodialysis patients with hyperphosphatemia, combining tenapanor with phosphate binders could improve phosphate control, compared with placebo, with diarrhea being a reason to discontinue in only 3.4% of patients. (87)
Further studies are needed to assess the tolerability and clinically relevant effects of phosphate binders and other phosphate-lower drugs in populations with diabetes. Vitamin D supplementation can result in lower PTH levels, while at the same time it would result in an increase in both phosphate and FGF23 levels, mainly in vitamin D-deficient patients. (88,89) This is likely caused by 1,25(OH)2-vitamin D interacting with the promotor region of the FGF23 gene, stimulating FGF23 production. At the same time, replenishing vitamin D in vitamin D-deficient patients may induce hyperphosphatemia through enhanced intestinal phosphate absorption. The resulting increase in phosphate may further boost FGF23 levels. (89) It has, however, been shown in a CKD rat model that calcitriol reduces expression of FGFR4 (one of the receptors for FGF23), and is subsequently able to reduce left ventricular hypertrophy. This suggests that the cardioprotective effects of calcitriol may be mediated by inhibition of the FGF23/FGFR4 pathway in the heart. (90)

In the era of monoclonal antibody therapy, it may seem desirable to target high FGF23 levels with anti-FGF23 antibodies. In fact, the anti-FGF23 monoclonal burosumab has recently been approved for use in adults and children with XLH, a rare disease characterized by an inappropriate overproduction of FGF23, leading to renal phosphate wasting. (91) Burosumab did not only result in lowering of FGF23 levels, it also resulted in the restoration of phosphate balance and improvement of bone abnormalities in children. (92) The question remains whether anti-FGF23 therapy would be suitable in other settings of increased FGF23, such as individuals with prediabetes and elevated FGF23. This would provide an opportunity to investigate the potential of reducing the risk of developing T2D. A warning signal was obtained from an animal study using another anti-FGF23 monoclonal in a rat CKD model: the net effect of anti-FGF23 treatment was reduced urinary phosphate excretion, hyperphosphatemia, increased aortic calcification and a high risk of mortality. (93)

Along the same lines, FGFR4 antagonists are being developed for treatment of XLH. This novel strategy uses a pan-FGF23 receptor inhibitor to block the FGF23 receptor and downstream signaling effects. A study in mice showed that using this FGFR4 inhibitor could indeed yield such effects; however, at the same time adverse effects were seen including hyperphosphatemia and a higher risk of calcification. (94) Thus, direct targeting of FGF23 or FGFR4 signaling may not be appropriate in individuals with T2D who are at risk for cardiovascular disease, and it may be preferable to target the triggers driving high FGF23 (such as phosphate) instead.

If indeed factors directly related to diabetes could increase FGF23, better glycemic control might stabilize or decrease FGF23 levels in persons with T2D. This
might be achieved by early initiation of basal insulin on top of glucose-lowering
drugs (27), increased physical activity (95), adherence to a healthier diet (96), and
continuous glucose monitoring. (97) Future studies should elucidate the effects of
improving glycemic control on FGF23 levels in persons with T2D.

In summary, the main findings of this thesis demonstrate a reciprocal interac-
tion between glucose and phosphate metabolism, whereby alterations in one
pathway exert influence on the other. Additionally, our studies offer substantial
evidence supporting the increased vulnerability of individuals with diabetes to
phosphate-induced vascular calcification. The studies from this thesis suggest that
aiming for better control of both glucose and phosphate metabolism could be help-
ful to improve cardiovascular outcomes in T2D. As such, the research described
in this thesis sets the stage for follow-up studies that will prospectively address
the potential beneficial effects of therapies that lower phosphate and FGF23 along
with better diabetes regulation, in an attempt to reverse the pandemic of T2D and
CKD we are facing today.
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