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A chronic high phosphate intake in mice is detrimental for bone health without major renal alterations

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Abstract: BACKGROUND Phosphate intake has increased in the last decades due to a higher consumption of processed foods. This higher intake is detrimental for patients with chronic kidney disease, increasing mortality and cardiovascular disease risk and accelerating kidney dysfunction. Whether a chronic high phosphate diet is also detrimental for the healthy population is still under debate. **METHODS** We fed healthy mature adult mice over a period of one year with either a high (1.2% w/w) or a standard (0.6% w/w) phosphate diet, and investigated the impact of a high phosphate diet on mineral homeostasis, kidney function and bone health. **RESULTS** The high phosphate diet increased plasma phosphate, parathyroid hormone (PTH) and calcitriol levels, with no change in fibroblast growth factor 23 levels. Urinary phosphate, calcium and ammonium excretion were increased. Measured glomerular filtration rate was apparently unaffected, while blood urea was lower and urea clearance was higher in animals fed the high phosphate diet. No change was observed in plasma creatinine levels. Blood and urinary pH were more acidic paralleled by higher bone resorption observed in animals fed a high phosphate diet. Total and cortical bone mineral density was lower in animals fed a high phosphate diet and this effect is independent of the higher PTH levels observed. **CONCLUSIONS** A chronic high phosphate intake did not cause major renal alterations, but affected negatively bone health, increasing bone resorption and decreasing bone mineral density.

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A chronic high phosphate intake in mice is detrimental for bone health without major renal alterations

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ABSTRACT

Background. Phosphate intake has increased in the last decades due to a higher consumption of processed foods. This higher intake is detrimental for patients with chronic kidney disease, increasing mortality and cardiovascular disease risk and accelerating kidney dysfunction. Whether a chronic high phosphate diet is also detrimental for the healthy population is still under debate.

Methods. We fed healthy mature adult mice over a period of one year with either a high (1.2 % w/w) or a standard (0.6 % w/w) phosphate diet, and investigated the impact of a high phosphate diet on mineral homeostasis, kidney function and bone health.

Results. The high phosphate diet increased plasma phosphate, parathyroid hormone (PTH) and calcitriol levels, with no change in fibroblast growth factor 23 levels. Urinary phosphate, calcium and ammonium excretion were increased. Measured glomerular filtration rate was apparently unaffected, while blood urea was lower and urea clearance was higher in animals fed the high phosphate diet. No change was observed in plasma creatinine levels. Blood and urinary pH were more acidic paralleled by higher bone resorption observed in animals fed a high phosphate diet. Total and cortical bone mineral density was lower in animals fed a high phosphate diet and this effect is independent of the higher PTH levels observed.

Conclusions. A chronic high phosphate intake did not cause major renal alterations, but affected negatively bone health, increasing bone resorption and decreasing bone mineral density.

KEY LEARNING POINTS

What is already known about this subject?

- Phosphate intake has increased in the last decades due to a higher consumption of processed food and changed food habits. Whether this leads to higher phosphate levels in blood is debated.
- High phosphate intake and/or increased phosphate levels in plasma have been associated to higher mortality and cardiovascular risk and accelerated loss of kidney function.
- The detrimental effect of a high phosphate diet on health is still under debate.

What this study adds?

- Using mice, we investigated the effect of a high phosphate intake without changing macronutrient composition on kidney and bone health over a long time-period.
- The study reveals that a high phosphate diet causes mild hyperfiltration but no further major alterations.
- A chronic high phosphate diet is detrimental for bone health probably due to the mild chronic acid load, decreasing bone mineral density and increasing bone resorption, in a PTH-independent manner.

What impact this may have on practice or policy?

- Recommendations on upper limit consumption on phosphate should take into account the detrimental effects on bone health.
- Regulation of phosphate additives in food labels should be revised for an appropriate assessment of phosphate dietary content.
- Reduction or exclusion of processed food intake may be beneficial not only for chronic kidney disease patients but also for the general population.

Keywords: parathyroid hormone, calcitriol, bone mineral density, high phosphate intake

INTRODUCTION

Phosphate is an essential constituent of living organisms. In mammals, phosphate is mainly found as hydroxyapatite in bones (85 %), and as phospholipids building the biological membranes in soft tissues. Other key functions of phosphate are its buffering capacity to maintain pH, as an integral part of adenosine triphosphate (ATP), as backbone of DNA and RNAs, as activator of diverse metabolites by phosphorylation and as an essential element in intracellular signaling [1]. Both, hyper- and hypophosphatemia are detrimental for health, affecting mainly the renal, cardiovascular and skeletomuscular systems. Phosphate metabolism is regulated by a complex network involving the skeleton, the kidneys, the intestine and the parathyroid glands and four major hormones, namely parathyroid hormone (PTH), calcitriol, fibroblast growth factor 23 (FGF23) and α Klotho [2]. The sodium-driven renal phosphate cotransporters NaPi-IIa and NaPi-IIc (SLC34A1 and SLC34A3) are regulated by PTH and FGF23 (and membrane bound α Klotho) and play a prominent role in controlling blood phosphate levels [3]. PTH and FGF23 also control calcitriol metabolism in the kidney by modulating the renal expression of 1α -hydroxylase (Cyp27b1) and 24-hydroxylase (Cyp24a1), the key enzymes in calcitriol synthesis [4]. Calcitriol modulates phosphate absorption in the intestine, and synthesis and secretion of FGF23 in bone and PTH in parathyroid glands [5].

Phosphate is widely distributed in food. Its bioavailability varies between 20 and 80%, being higher in food of animal origin and lower in vegetables derived food [6]. Inorganic phosphate, present in processed food as additive and preservative, has a higher bioavailability (over 80 %). Assessment of dietary phosphate is often underestimated, due mostly to the increased consumption of processed food where phosphate content is not required to be reported. Yet, several studies suggest that the average intake in various countries varies between 800 and 1'600 mg/day, with individuals reporting very high intakes up to 5'000 mg/day [7], and this phosphate intake is increasing since 1970 [8]. Whereas the US recommended dietary allowance (RDA) for phosphate is 700 mg/day [9], a twofold higher average intake has been reported in this country [10]. Therefore, there is an increasing concern whether a chronic high phosphate intake may be detrimental for health [11, 12]. Phosphate overload is indeed detrimental for individuals already suffering from diseases such as chronic kidney disease (CKD). In CKD patients, high phosphate serum levels are associated with higher mortality risk and a higher incidence of cardiovascular disease [13-15]. Therefore, restricted phosphate consumption with minimal contribution from processed food is recommended in these patients [16]. Furthermore, several association studies in humans suggest that healthy individuals with plasma phosphate levels in the normal range, but in the higher percentile, show also accelerated loss of kidney function [17-19]. Phosphate intake above 1'400 mg/day has been associated with higher

overall cause mortality, and elevated phosphate serum levels with a higher incidence of cardiovascular disease in the general population [15, 20]. However, an expert panel from the European Food and Safety Administration (EFSA) aiming to define an acceptable daily phosphate intake in the general population, has recently concluded that the current studies are not conclusive about the potential toxicity [21].

Here, we investigated if a twofold higher phosphate intake over a prolonged period (over one year) was detrimental for health of mature adult mice, focusing on the renal-bone axis. The aim of the study was to analyze the net effect due exclusively to phosphate consumption without modifying the macronutrient composition of the diets.

MATERIALS AND METHODS

Mice and diets

C57BL/6JRj male mice aged 26 weeks were obtained from Janvier Labs (Le Genest-Saint-Isle, France), split randomly in two groups and fed for 58 weeks (protocol 1) or 16 weeks (protocol 2) on either a customized standard or high phosphate diet: 0.6 % (SP) or 1.2 % (HP) w/w phosphorus (as calcium or potassium salts), 1% w/w calcium, and 600 IU/kg cholecalciferol (ssniff Spezialdiäten GmbH, Germany). A third group of animals (protocol 3) was bred at the University of Zurich. PTH knockout [22] and wildtype littermates maintained with 2% calcium gluconate in water and aged 6-11 weeks were fed for 4 weeks either the customized standard or a very high phosphate diet (2 % (H2P) w/w phosphorus (as calcium and potassium salts), 1% w/w calcium, and 600 IU/kg cholecalciferol; ssniff Spezialdiäten GmbH, Germany). All diets contained 20 % energy equivalents in the form of casein. Mice were placed in metabolic cages (Tecniplast Inc., Italy) for 24 h for urine (under mineral oil) and feces collection. The animals were maintained at 22 ± 2 °C and a 12:12 h light/dark cycle with access to tap water and diet ad libitum. Further details for protocols 1-3 are included in the supplementary data. All procedures applied throughout this study were conducted according to the Swiss animal welfare laws and guidelines for animal care and approved by the Zurich Veterinary Office under the reference numbers 05/2013 and 156/2016.

Urine and blood parameters

Phosphate, calcium and creatinine in 24-hour urine and plasma, blood urea nitrogen, urine urea nitrogen, ammonium in 24-hour urine, and alkaline phosphatase in plasma was measured in the Zurich Integrative Rodent Physiology (ZIRP) platform of the University of Zurich using the UniCel DxC 800 Synchron System (Beckman Coulter, USA). Bicarbonate, the calculated anion gap, pH and partial pressure of carbon dioxide in plasma was determined with the ABL 77 Radiometer (Radiometer, Denkmär). Urine pH was measured using a microHmeter. Intact FGF23 and PTH in plasma were quantified using the FGF23 ELISA Kit and the mouse intact PTH ELISA kit (Immunotopics International, USA). Calcitriol was quantified using the 1,25-Dihydroxy Vitamin D RIA (Immunodiagnostic System, Frankfurt am Main, Germany). Quantification of DPD and NGAL in urine was performed using the MicroVue DPD EIA kit (Quidel Corporation, USA) and the Lipocalin-2/NGAL Kit (R&D systems, USA).

Gene and protein expression analysis

Total RNA extraction, cDNA synthesis and semiquantitative RT-PCR were performed as described previously [23]. *Cyp27b1* and *18S* gene expression was detected using primers and probes from

Applied Biosystem (USA; Cat. No. 4351372 and 4310893). Protein isolation and western blot was performed as described previously [23]. The primary and secondary antibodies used are listed in Table S2.

Kidney, bone and respiratory function phenotyping

Glomerular filtration rate (GFR) was measured by a non-invasive-clearance measurement using FITC-Sinistrin (Fresenius Kabi, Austria) and the NIC-Kidney device (Mannheim Pharma & Diagnostic, Germany), calcium content in kidney was determined in renal homogenates using the QuantiChrom Calcium assay kit (BioAssay Systems, USA) and bone mineral density was determined in isolated femurs with the Quantum FX microCT Imaging System (PerkinElmer, USA), as described previously [23]. Whole-body plethysmography was determined using EMKA Technologies (USA), as described previously [24].

Statistics

Statistical analyses were performed using the R statistical language 4.0.2 (R Studio Inc, USA) and Python programming language 3.7.3 (Python Software Foundation, USA). Major outliers defined as values lower than $1stQ - 3 * IQR$ or greater than $3rdQ + 3 * IQR$ (Q: quartile and IQR: interquartile range) were removed from the analysis. Statistical significance, defined as $P < 0.05$, was calculated by a two-tailed Student's t-test or two-way ANOVA followed by Tukey HSD test for multiple comparisons. Data are presented as mean $\pm$ SEM.

RESULTS

Frailty and chronic high phosphate intake

In protocol 1, 12 male mice aged 26 weeks were fed a HP diet (1.2 % w/w P) and age-matched 10 male mice were fed a SP diet (0.6 % w/w P) for 58.24 ± 0.73 weeks. Mice showed similar food intake levels under both diets. At the end of the experiment, most animals showed the typical signs of aged mice (mean age 84.24 ± 0.73 weeks), like hair greying and thinning. Four animals had to be discarded from the analysis. Three of them (one under a HP diet) looked too frail for experiments; the fourth one (fed a HP diet) had no apparent signs of frailty but after sacrifice, we observed that the animal had very fragile bones and very high phosphate, FGF23 and PTH plasma levels in plasma (data not shown).

Phosphate homeostasis after a chronic high phosphate intake

Administration of HP diet during 58 weeks increased phosphate levels in plasma as well as urinary phosphate and calcium excretion, whereas calcium levels in plasma, and phosphate and calcium excreted in feces were similar between the diets (Figure 1A-D, Figure S1A-B). Phosphate balance was positive and significantly increased in animals consuming a high phosphate diet (Fig S1C). Intact FGF23 showed a tendency to be higher in animals consuming a HP diet (Fig. 1E; P-value = 0.058), and no altered protein expression of its co-receptor α Klotho was observed in kidney (Figure S1D-E). PTH was clearly increased after a chronic HP diet (Figure 1F). This increase is most likely responsible for the lower renal NaPi-IIa protein expression observed in animals consuming a HP diet (Figure 1G-H). Renal NaPi-IIc protein expression showed a tendency to be lower after a HP diet (Figure S1F-G). We also observed increased calcitriol levels along with higher renal mRNA levels of the key enzyme involved in calcitriol synthesis, *Cyp27b1*, in animals consuming a HP diet (Figure 1I-J). The inactivating enzyme of calcitriol, *Cyp24a1*, and the calcitriol receptor *Vdr* showed no difference in renal protein expression due to the phosphate content in diet (Figure S1H-J).

Kidney function after a chronic high phosphate diet

GFR was probably similar in animals consuming both diets for 58 weeks (Figure 2A). Plasma creatinine was similar, whereas animals consuming a HP diet showed lower blood urea nitrogen (BUN) levels (Figure 2B-C). No difference was observed in the volume of urine excreted in 24 h between the diets (Figure S2A), but urea clearance was significantly higher in mice fed a HP diet (Figure 2D). Neutrophil gelatinase-associated lipocalin (NGAL) in urine, a marker of kidney damage, was unaffected by a HP diet (Figure 2E). As high plasma phosphate leads to hyperparathyroidism, we looked for renal calcium content, which was similar between diets (Figure 2F). After 58 weeks of

feeding a HP diet, the animals showed similar systolic blood pressure than animals fed a SP diet (Figure S2B).

Next, we examined kidney function in animals fed only for 16 weeks (protocol 2) a HP diet to test if a shorter exposure to HP will influence kidney parameters. These animals also had increased plasma and urine phosphate levels after a HP diet (Figure 3A-B). GFR, creatinine in plasma, BUN, urinary volume excreted in 24 hours, urea clearance and urinary NGAL excretion, were all similar between animals consuming a HP and SP diet (Figure 3C-G, Figure S2C).

Chronic high phosphate diet decreases bone mineral density

We observed increased plasma alkaline phosphatase levels in animals fed a chronic a HP diet for 58 weeks (Figure 4A), indicating bone alterations. Indeed, mice fed a HP diet had decreased bone mineral density (BMD; Figure 4B). Cortical BMD was also decreased after a chronic HP diet, whereas trabecular BMD was similar between animals fed HP and SP diets (Figure 4C-D). Urinary excretion of deoxypyridinolin (DPD), a bone resorption marker, was clearly increased in animals fed a HP diet (Figure 4E).

The decrease in cortical bone mineral density is probably not due to high PTH levels. We challenged another group of animals (protocol 3), namely PTH knockout mice with a higher phosphate diet (H2P: 2 % w/w) during four weeks. BMD and trabecular BMD were similar in all animals (Figure 5A-B). Cortical BMD was significantly decreased in animals fed a HP, with no differences between genotypes (Figure 5C).

A high phosphate diet increases the acid load

Urinary ammonium excretion was highly increased in animals fed a HP diet for 58 weeks (Figure 56A). Phosphoenol-pyruvate carboxykinase (Pepck) and phosphate dependent glutaminase (Pdg) are two enzymes involved in ammoniagenesis. Protein expression of these enzymes was not altered due to the phosphate content in the diet (Figure 6B-D).

The increased urinary excretion of ammonium is already observed after 16 weeks on a HP diet (protocol 2, Figure 7A). In these animals, we further characterized their acid-base balance. Urinary pH was more acidic after a HP diet (Figure 7B). Blood gas analysis showed that a HP diet decreases also blood pH with no difference in blood bicarbonate concentration (Figure 7C-D). The calculated anion gap and partial pressure of carbon dioxide in blood were similar regardless of the phosphate content in the diet (Figure S3A-B). Respiratory function assessed by whole-body plethysmography was not altered (Figure 7E, Figure S3C-D).

DISCUSSION

Whether consuming chronically a high phosphate diet is harmful for healthy individuals is under debate [11, 12, 21]. This debate is of crucial interest as phosphate consumption has increased over the last decades due to a higher intake of processed food that contain phosphate as an additive [6, 8]. In this study, we used mature adult mice and fed them over one year (protocol 1) with a high phosphate diet, twofold the standard phosphate diet trying to mimic the reported intake in the human population. Importantly, both the long term and short term chronic high phosphate load increased phosphate levels in plasma. This increase happened although the animals had normal kidney function and were capable to increase the urinary excretion of phosphate by reducing NaPi-IIa protein levels. Of the two phosphaturic hormones, FGF23 and PTH, PTH has being assigned a more predominant role in the acute adaptation to a high phosphate load [25-27]. Here we found that after a long term high phosphate intake, PTH levels in plasma increased markedly, suggesting also a role of PTH in the chronic adaptation to a high phosphate load/intake. The increased PTH levels may also be responsible for the higher calcitriol levels due to enhanced synthesis and for increased phosphate excretion in urine via down regulation of the active phosphate transporter NaPi-IIa [3, 5]. Yet, the increase in the calcitriol levels is somehow puzzling as the mice have increased phosphate levels in plasma and show normocalcemia. This increase may also be a consequence of the enhanced acid load (see below), as observed in humans where metabolic acidosis induced calcitriol production [28].

High phosphate and high calcitriol levels in plasma have been described as the main inductors of FGF23 production [29]. Yet, after one year of chronic high phosphate intake, we did not observe increased intact FGF23 levels in plasma. In humans an enteral and parenteral high phosphate administration over 36-hours elevated C-terminal FGF23 levels [30]. Two further studies in humans showed increased FGF23 levels in plasma after a high phosphate intake when compared to a low phosphate intake [31, 32]. In mice, higher phosphate intake (1.65 % P w/w and 2 % P w/w instead of 1.2 % P w/w) during 5 days and 4 weeks, respectively, increased FGF23 levels in plasma [33, 34]. Therefore, here a transient increase of FGF23 may occur but during the long-term phosphate feeding adaptive changes may take place that allow FGF23 to return to normal levels or that prevent maintenance of higher FGF23 levels. Whether these inappropriately normal FGF23 levels (with regard to blood Pi levels) are part of a maladaptive process or are beneficial in an attempt to not further aggravate bone health by suppressing PTH and calcitriol remains to be resolved.

To our knowledge, this is the first study that analyzed how a high phosphate intake affects kidney function in healthy animals over a long period. A large retrospective longitudinal cohort study in humans suggested a higher risk for end-stage renal disease in subjects with higher plasma phosphate levels [19]. However, a substantial part of these subjects had hypertension or diabetes as cofounding conditions and phosphate intake data were not assessed. Our data indicate that the one year a-chronic high phosphate intake increased urea clearance suggesting a mild hyperfiltration. High filtration rates have been associated with a higher risk to progress to kidney disease in patients with diabetes or hypertension [35]. On the other hand, no signs of nephrocalcinosis, despite the high PTH levels, were detected here. Of note, high phosphate intake is usually linked to high protein (meat) intake [6, 16]. In this study we did not change the macronutrient composition and looked exclusively for the effects of high phosphate consumption. Moreover, a high phosphate intake is often associated to a higher consumption of processed food and low income, which may have a synergistic effect on kidney health [11].

Phosphate is an inorganic anion. Thus, a two-fold higher ingestion of phosphate increases by two-fold the potential renal acid load (PRAL) [36, 37]. The kidney plays a major role in maintaining acid-base balance by three main mechanisms: by excreting acids as ammonium or titrable acids, by increasing the reabsorption of bicarbonate and by inducing the synthesis and restoration of bicarbonate levels during ammoniumgenesis [38]. Besides lowering blood pH, the chronic higher acid load led to a lower urinary pH and a remarkably higher urinary excretion of phosphate and ammonium, with no change in PDG and PEPCK levels. These enzymes are key regulators of ammoniumgenesis and are upregulated in metabolic acidosis [39, 40]. However, regulation may also occur on the posttranslational level.

In the shorter phosphate loading protocol 2, we showed that a chronic ingestion of a two-fold higher phosphate diet led to a decrease in blood pH, indicating that acid-base balance could not be fully maintained. However, no changes in bicarbonate levels were observed suggesting long-term adaptation. This chronic acid load did not caused changes in partial carbon dioxide pressure and the respiratory function, suggesting the absence of respiratory compensation.

The bone plays a crucial role in maintaining acid-base balance during prolonged acid load, reflected by markedly high urinary calcium loss [41]. Indeed, after the one year high phosphate diet the animals showed high urinary calcium excretion with no changes in plasma calcium levels. DPD in urine, a marker of bone resorption, and alkaline phosphate in blood, a marker of bone disorders, were clearly increased in animals consuming a high phosphate diet over one year. Moreover, as assessed by microcomputer tomography a decrease in total and cortical BMD was detectable after a

chronic high phosphate load. Importantly, this loss of bone mineral is not dependent on the increased PTH levels. PTH is able to further stimulate osteoclasts activated by acidosis [42], and hyperparathyroidism adversely impacts bone health [43]. Yet, here we found that only the phosphate content and not ablation of PTH function led to BMD loss. Therefore, the chronic and continuous acid load may be responsible for the loss in bone mineral density observed after a high phosphate diet, as reported in other settings with metabolic acidosis [44], and therefore detrimental for health. The role of PTH in bone loss may be different from CKD where a role of PTH in promoting bone loss has been suggested [45].

In conclusion, we show in mice that a high phosphate content diet consumed over a long time period increases the acid load in the organism with detrimental consequences on bone health. Bone resorption is activated in order to maintain acid-base balance and hypercalciuria is detectable. The phosphate excess causes also an increase in plasma phosphate which may contribute to hyperparathyroidism. High PTH levels in plasma may increase calcitriol synthesis and levels in plasma and promote urinary phosphate excretion. FGF23 levels are inappropriately normal. These alterations in mineral homeostasis and the mild acidosis do not seem to impair renal function but causes mild hyperfiltration. Therefore, chronic high phosphate intake in healthy animals is detrimental for bone health. As high phosphate consumption in humans is usually associated to higher protein consumption, the combined higher intake of these nutrients may be responsible for the associated higher risk of loss of renal function reported.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they are not aware of any conflicts of interest and that the results presented in this paper have not been published previously in whole or part, except in abstract format. CAW reports honoraria and grants from Bayer, Medice, Kyowa Kirin, and Chugai.

AUTHORS' CONTRIBUTIONS

C.B., E.-M. P.-A. and N.G. conducted the experiments. M.U., S.B. and A.D. conducted the experiments, analysed the data and interpreted the results. N.H. and C.A.W. designed the experiments and interpreted the results. I. R.-A. designed the experiments, conducted the experiments, analysed the data, interpreted the results and wrote the manuscript. All authors read the manuscript, contributed to editing, and approved the final version.

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DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material.

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LEGENDS TO FIGURES

FIGURE 1. Phosphate and calcium balance after a high phosphate diet for 58 weeks. **A,C** Phosphate and calcium levels in plasma (SP n = 8, HP n = 9-10). **B,D** Phosphate and calcium levels in 24 hours urine corrected by creatinine (SP n = 8, HP n = 9-10). **E-F** Intact FGF23 and PTH levels in blood (SP n = 8, HP n = 10). **G-H** Representative western blot and densitometry analysis of NaPi-IIa and actin protein expression in the kidney (SP n = 6, HP n = 7). **I** Calcitriol levels in blood (SP n = 8, HP n = 10). **J** mRNA relative expression of *Cyp27b1* in kidney using *18S* as housekeeping gene (SP n = 7, HP n = 10). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. Differences due to diet: *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001.

FIGURE 2. Effects of a high phosphate diet for 58 weeks on kidney function parameters. **A** Glomerular filtration rate (GFR) assessed by the elimination kinetics of FITC-sinistrin (SP n = 6, HP n = 5). **B-C** Creatinine in plasma and blood urea nitrogen (BUN) (SP n = 8, HP n = 10). **D** Urea clearance (Cl) calculated as (urea in 24 hours urine * volume urine in 24 hours)/BUN (SP n = 7, HP n = 10). **E** Neutrophil gelatinase-associated lipocalin (NGAL) in 24 hours urine normalized by creatinine (SP n = 5, HP n = 8). **F** Calcium content in kidney normalized by kidney weight (SP n = 7, HP n = 10). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. Differences due to diet: *P-value < 0.05.

FIGURE 3. Effects of a high phosphate diet for 16 weeks on kidney function parameters. **A** Phosphate levels in plasma (SP n = 18, HP n = 18). **B** Phosphate in 24 hours urine corrected by creatinine (SP n = 7, HP n = 8). **C** Glomerular filtration rate (GFR) assessed by the elimination kinetics of FITC-sinistrin (SP n = 9, HP n = 8). **D-E** Creatinine in plasma and blood urea nitrogen (BUN) (SP n = 10, HP n = 10). **F** Urea clearance (Cl) calculated as (urea in 24 hours urine * volume urine in 24 hours)/BUN (SP n = 7, HP n = 8). **G** Neutrophil gelatinase-associated lipocalin (NGAL) in 24 hours urine normalized by creatinine (SP n = 7, HP n = 6). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. Differences due to diet: **P-value < 0.01, ***P-value < 0.001.

FIGURE 4. A high phosphate diet for 58 weeks is detrimental for bone health. **A** Alkaline phosphatase levels in plasma in wildtype animals fed for 58 weeks a high or a standard phosphate diet (SP n = 6, HP n = 9). **B-D** Bone mineral density (BMD), cortical and trabecular BMD in wildtype animals fed for 58 weeks a high or a standard phosphate diet (SP n = 6, HP n = 7). **E** Urinary excretion of deoxypyridinolin (DPD) normalized by creatinine in 24 hours urine samples of wildtype animals fed for 58 weeks a high or a standard phosphate diet (SP n = 7, HP n = 10). SP: 0.6 % P w/w

diet, HP: 1.2 % P w/w diet. Differences due to diet: *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001.

FIGURE 5. A high phosphate diet for 4 weeks decreases bone mineral density in wildtype and PTH KO animals. A-C Bone mineral density (BMD), cortical and trabecular BMD in PTH KO and wildtype animals fed for 4 weeks a higher or a standard phosphate diet (Two-way ANOVA P-value due to diet: $8E-05$; WT SP n = 8, WT H2P n = 8, KO SP = 7, KO H2P = 8). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. H2P: 2 % P w/w diet. WT: wildtype mic3, PTH KO: PTH knockout mice. Differences due to diet: *P-value < 0.05, **P-value < 0.01.

FIGURE 6. A high phosphate diet for 58 weeks increases urinary ammonium excretion. A Ammonium excreted in 24 hours urine normalized by creatinine (SP n = 7, HP n = 10). **B-D** Representative western blot and densitometry analysis of phosphoenol-pyruvate carboxykinase (Pepck), phosphate dependent glutaminase (Pdg) and actin protein expression in kidney samples of wildtype animals fed for 58 weeks a high or a standard phosphate diet (SP n = 6, HP n = 8). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. Differences due to diet: ***P-value < 0.001.

FIGURE 7. A high phosphate diet for 16 weeks increases the acid load. A Ammonium excreted in 24 hours urine normalized by creatinine in wildtype animals fed for 16 weeks a high or a standard phosphate diet (SP n = 7, HP n = 10). **B** Urine pH in wildtype animals fed for 16 weeks a high or a standard phosphate diet (SP n = 7, HP n = 10). **C-D** Blood pH and bicarbonate concentration in blood in wildtype animals fed for 16 weeks a high or a standard phosphate diet (SP n = 9, HP n = 9). **E** Respiratory function assessed by whole-body plethymography (SP n = 10, HP n = 10). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet. Differences due to diet: *P-value < 0.05, ***P-value < 0.001.

Figure 1

Figure 2

Figure 3

A

B

C

D

E

F

G

Figure 4

Figure 5

A

B

C

Figure 6

Figure 7

A

B

C

D

E

SUPPLEMENTARY MATERIAL

A chronic high phosphate intake in mice is detrimental for bone health without major renal alterations

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SUPPLEMENTARY FIGURES

FIGURE S1. Phosphate and calcium metabolism after a high phosphate diet for 58 weeks. A Phosphate excreted in feces corrected by food intake (SP n = 8, HP n = 10). **B** Calcium excreted in feces corrected by food intake (SP n = 8, HP n = 8). **C** Phosphate balance calculated as phosphate consumed minus phosphate excreted in feces and urine (SP n = 7, HP n = 8). **D-E** Representative western blot and densitometry analysis of α Klotho and actin protein expression in the kidney (SP n = 6, HP n = 8). **F-G** Representative western blot and densitometry analysis of NaPi-IIc and actin protein expression in the kidney (SP n = 6, HP n = 6). **H-J** Representative western blot and densitometry analysis of Cyp24a1, Vdr and actin protein expression in the kidney (SP n = 6, HP n = 8). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet.

FIGURE S2. Urinary volume and blood pressure after a standard and high phosphate diet. **A** Urine volume (V) excreted in 24 hours after 58 weeks fed a high and standard phosphate diet (SP n = 8, HP n = 9). **B** Systolic blood pressure (BP) assessed by the tail-cuff in animals fed a high and a standard phosphate diet for 58 weeks (SP n = 7, HP n = 9). **C** Urine volume (V) excreted in 24 hours after 16 weeks fed a high and standard phosphate diet (SP n = 10, HP n = 10). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet.

FIGURE S3. Blood gas analysis parameters and respiratory function after a high phosphate diet for 16 weeks. **A-B** Blood gas analysis parameters, the calculated anion gap and the partial pressure of carbon dioxide (SP n = 9, HP n = 9). **C-D** Respiratory function assessed by whole-body plethysmography (SP n = 10, HP n = 10). SP: 0.6 % P w/w diet, HP: 1.2 % P w/w diet.

SUPPLEMENTARY MATERIALS AND METHODS

Phosphate and calcium in feces

Feces were dried for 3 days at 65°C, dissolved in 0.6 M HCl for 3 days and centrifuged. In the supernatant phosphate was measured according to the Fiske Subbarow method [1], and calcium using the QuantiChrom Calcium assay kit (Bio-Assay Systems, USA).

Systolic blood pressure

Systolic blood pressure was analyzed using the tail-cuff method and the BP-2000 Series II blood pressure analysis system (Visitec System Apex, USA). Blood pressure was measured on 5 consecutive days at the same time during the morning and only the last day records were taken for analysis.

Animal protocols

Group 1: C57BL/6Jrj male mice aged 26 weeks and obtained from Janvier Labs (Le Genest-Saint-Isle, France), were split randomly in two groups and fed for an average of 58 weeks on either a customized standard or high phosphate diet: 0.6 % (SP) or 1.2 % (HP) w/w phosphorus (as calcium or potassium salts), 1% w/w calcium, and 600 IU/kg cholecalciferol (ssniff Spezialdiäten GmbH, Germany). Animals were analyzed at the end of the feeding challenge over three consecutive weeks for systolic blood pressure by the tail-cuff method, for glomerular filtration rate by a non-invasive-clearance measurement using FITC-Sinistrin, for bone mineral density by microCT and put in metabolic cages for 24 h urine and feces collection before sacrificing the animals.

Group 2: C57BL/6Jrj male mice aged 26 weeks and obtained from Janvier Labs (Le Genest-Saint-Isle, France), were split randomly in two groups and fed for 16 weeks on either a customized standard or high phosphate diet: 0.6 % (SP) or 1.2 % (HP) w/w phosphorus (as calcium or potassium salts), 1% w/w calcium, and 600 IU/kg cholecalciferol (ssniff Spezialdiäten GmbH, Germany). Animals were analyzed at the end of the feeding challenge in two consecutive weeks for glomerular filtration rate by a non-invasive-clearance measurement using FITC-Sinistrin and put in metabolic cages for 24 h urine before sacrificing the animals.

Group 3: PTH knockout [2] and wildtype littermates aged 6-11 weeks and obtained from breeding at the University of Zurich and maintained with 2% calcium gluconate in water, were fed for 4 weeks either the customized standard or a very high phosphate diet (2 % (H2P) w/w phosphorus (as calcium or potassium salts), 1% w/w calcium, and 600 IU/kg cholecalciferol; ssniff Spezialdiäten GmbH, Germany). At the end of the for weeks the animals bone mineral density in these mice was analyzed by microCT.

In all protocols, diets contained 20% of energy as protein under the form of casein.

SUPPLEMENTARY TABLES

TABLE S1. Commercial Kits

Name	Company	Catalog Number
Mouse/Rat FGF-23 (Intact) Kit	Immutopics International	60-6800
Mouse intact PTH ELISA kit	Immutopics International	60-2305
MicroVue DPD EIA Kit	Quidel Corporation	8007
Lipocalin-2/NGAL Kit	R&D Systems	MLCN20
1,25-Dihydroxy Vitamine D RIA kit	Immunodiagnostic System	AA-54F1

TABLE S2. Antibodies

Name	Company	Catalog Number
α -Actinin	Sigma	A5044, Clone DM-75.2
Cyp24a1	Proteintech	21582-1-AP
α Klotho	Transgenic Inc.	KO603, Clone KM2076
NaPi-IIa	Laboratory H. Murer, University of Zurich (CH) [3]	N/A
NaPi-IIc	Laboratory of C. Wagner, University of Zürich (CH) [4]	N/A
Pdg	Laboratory N. Curthoys, Colorado State University (USA)	N/A
Pepck	Cayman Chemical	10004943
VDR (D-6)	Santa Cruz Biotechnology	sc-13133
Anti-Mouse IgG (H+L), HRP Conjugate	Promega	W402B
Anti-Rabbit IgG (H+L), HRP Conjugate	Promega	W401B

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