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Review

New concepts in phosphorus homeostasis and its impact on renal health with particular reference to the cat

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ABSTRACT

New discoveries relating to phosphorus homeostasis include the hormones fibroblast growth factor-23 and klotho produced by bone and kidney. These hormones, together with novel understanding of how calcium and phosphate ions are carried in colloidal form as calciprotein particles, have changed our view of how phosphorus is regulated. Recognition that high dietary intake of inorganic forms of phosphorus in humans is a risk factor for both cardiovascular and renal diseases have led to re-examination of the impact of inorganic sources of phosphorus in prepared cat foods on renal health. Data suggest that when homeostatic mechanisms lead to proximal tubular (S3 segment) phosphate concentrations exceeding 3.25 mmol/L for a significant part of the day, tubular stress and structural kidney damage ensues. Recent experimental rodent studies support the concept that calciprotein particles form in the proximal tubule at these prevailing phosphate concentrations and trigger proximal tubular damage. Long-term feeding studies in cats suggest that carefully formulated prepared diets containing 1 g/Mcal of inorganic phosphorus (in the form of sodium tripolyphosphate or potassium monophosphate and pyrophosphate), resulting in estimated tubular phosphate concentrations < 2.5 mmol/L can be fed to healthy adult cats without detectable adverse effects on renal health.

1. Introduction

Although phosphorus is a major element in mammals, regulation of serum phosphate concentration by specific phosphotonins has only started to be unravelled in the last 25 years. Discovery of klotho (Kuro-o et al., 1997) and Fibroblast Growth Factor 23 (FGF23; ADHR Consortium, 2000) and the realisation that these two factors work together (klotho is an essential co-factor for Fibroblast Growth Factor (FGF)-receptor signalling by FGF23) to regulate serum phosphate (Kurosu et al., 2006; Urakawa et al., 2006) were major milestones in understanding phosphorus homeostasis. Klotho and FGF23 deficiency both lead to hyperphosphataemia, premature aging, and soft tissue calcification (Nakatani et al., 2009). Thus, these two mediators are essential to prevent soft tissue mineralisation during normal skeletal growth and turnover.

Communication between bone (osteocytes/osteoblasts secrete FGF23), kidney (source of circulating klotho), parathyroid gland, and intestine is essential for serum phosphate regulation. Furthermore, this network needs to integrate with systems regulating serum calcium. Table 1 summarises our current understanding of how these body systems work together to regulate phosphorus.

2. Calciprotein particles

A recent discovery that has impacted on the integration of calcium and phosphorus homeostasis is that of nanoparticles, which carry circulating calcium and phosphate ions in colloidal suspension rather than in solution (Fig. 1; Smith et al., 2020). Coined calciprotein particles (CPP) because of their similarity to lipoprotein particles, fetuin-A, the particle's core protein, binds calcium and phosphate ion complexes and prevents crystal formation. CPPs initially form as calciprotein monomers (CPM; 8–10 nm in diameter) and aggregate to form primary (amorphous) CPPs (35–100 nm in diameter). It seems likely that CPMs are the most abundant particles formed in the circulation in vivo followed by primary CPPs. Secondary (crystalline) CPPs (100–250 nm in diameter) are unlikely to form in blood of healthy mammals but are detected in the sera of CKD patients receiving dialysis for chronic kidney disease (CKD), where their presence is associated with mortality risk (Chen et al., 2021).

Primary CPPs are likely to form spontaneously at sites of bone

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Table 1
Summary of current knowledge of the network of factors produced by bone, kidney, intestine and parathyroid gland which regulate serum phosphate. References to statements relating to phosphate regulation are provided. For a full review of calcium regulation see Tang et al. (2021).

	Hormone (s) produced	Factors regulating / modulating	Hormone action on tissue with effects (direct or indirect) on serum phosphate	References
Bone	FGF23	Calciprotein particles[a] or serum phosphate via FGF1R[b] PTH[c] and calcitriol[d] increase FGF23 expression	PTH[e] has both anabolic (at low concentration) and catabolic (at high concentration) effects on bone, in the latter case releasing calcium and phosphate ions Calcitriol[f] has endocrine and autocrine anabolic effects on bone. Also has catabolic effects at high concentrations and is synergistic with high concentrations of PTH Calcitonin[g] reduces osteoclast numbers, reducing basal and stimulated bone resorption FGF23[h] – role in bone turnover not understood but shown to suppress osteoblast differentiation and matrix mineralization	[a]Akiyama et al. (2020) [b]Takashi et al. (2019) [c]Lavi-Moshayoff et al. (2010) [d]Kolek et al. (2005) [e]Dobnig and Turner (1997) [f]Kondo et al., 2004 [g]Chambers et al. (1986) [h]Wang et al. (2008)
Intestine	Proposed unidentified factors communicating between intestine and kidney[i]	Not known – proposed intestinal phosphate sensor[i,j]	Unknown factor(s) communicates between intestine and kidney, upregulating urinary phosphate excretion when intestinal phosphate concentration is high[i] Calcitriol[k] upregulates sodium-dependent phosphate transporters NaPi2b in the intestine, regulating the transcellular route of phosphate absorption. Also increases calcium transcellular transport. At high intestinal phosphate concentrations, paracellular passive transfer occurs[l] Other hormone effects are indirect via calcitriol (see under kidney)	[i]Berndt et al., 2007 [j]Slatapolsky, 2011 [k]Marks et al. (2006) [l]Marks et al. (2015)
Kidney	Activates calcidiol (via activity of 1-α-hydroxylase) Releases klotho either to act locally within the kidney or to add to the soluble klotho pool	PTH upregulates calcitriol production[m] FGF23 down-regulates calcitriol production[n] (upregulates 24-hydroxylation of calcidiol) Regulators of klotho release from the kidney are poorly understood. Activators of CaSR (which co-localises with klotho in distal tubule) release klotho[o]	PTH[p] and FGF23[q] – phosphaturic effect (via down regulation of sodium-dependent phosphate co-transporter NaPi2a/c expression in the kidney) enhancing phosphate excretion PTH and calcitriol increase calcium absorption (distal tubules) Klotho production by distal tubule is needed for FGF23 phosphaturic effect[r]; also has direct effect on calcium reabsorption via calcium channel protein TRPV5	[m]Zierold et al. (2003) [n]Shimada et al. (2004) [o]Yoon et al. (2021) [p]Caverzasio et al. (1986) [q]Segawa et al. (2003) [r]Olauson et al. (2012)
Parathyroid gland	Releases parathyroid hormone	CaSR regulates PTH secretion (low ionised calcium relieves parathyroid gland from CaSR-mediated inhibition of PTH secretion) CaSR is inactivated by phosphate binding (proposed as phosphate sensing mechanism of parathyroid gland; explaining direct effect of high phosphate on PTH secretion)[s]	FGF23[t] and calcitriol[u] inhibit PTH secretion (negative feedback) Serum phosphate increases PTH secretion via an inactivating effect on the CaSR[s]	[s]Centeno et al., 2019 [t]Krajisnik et al. (2007) [u]Silver et al. (1986)

CaSR, calcium sensing receptor; FGF1R, fibroblast growth factor receptor 1; FGF23, fibroblast growth factor-23; PTH, parathyroid hormone; TRPV5, Transient receptor potential cation channel subfamily V member 5.

turnover and following phosphorus ingestion, particularly when a high phosphorus diet is fed. Rats fed a high phosphorus diet for 10 weeks showed an increase in serum primary CPPs without demonstrating an increase in serum phosphate concentration (Smith et al., 2017). Similarly, diabetic patients without CKD showed diurnal fluctuations in CPPs, increasing following a meal with no concomitant increase in serum phosphate (Yamada et al., 2018). Primary CPPs' physiological importance is underlined by recent findings that osteocytes/osteoblasts sense these particles and increase FGF23 expression in response to exposure to increasing concentrations of these particles (Akiyama et al., 2019). Acute phosphorus administration by oral gavage in mice led to a modest increase in serum CPPs (detected 2 h post-gavage) followed by a transient increase in FGF23 mRNA expression in bone (4 h post-gavage), and a delayed rise (25% increase) in circulating intact FGF23 (8 h post-gavage). Continuous high inorganic phosphorus feeding for 2 days led to a 2-fold increase in CPPs and a 3–4-fold increase in circulating intact FGF23 concentration (Akiyama et al., 2019). While this evidence is compatible with the observation that circulating intact FGF23 concentrations can be stimulated through CPP formation, and thus depends on carriage of both phosphate and calcium ions in colloidal form, the physiological relevance of the level of dietary phosphorus supplementation is questionable. Further studies are necessary to determine the mechanism(s) by which increased dietary phosphorus leads to raised circulating intact FGF23 concentrations.

The above mechanism, by which CPPs regulate FGF23, has been reproduced in cell culture using UMR106 osteoblasts that, when exposed to synthetic CPPs, increase FGF23 expression and secretion (after 8 h) without changes in phosphate or calcium ion concentration in the growth medium. Furthermore, in vivo studies (in mice) and in vitro studies using the same cell line have suggested that direct phosphate ion sensing by osteoblasts occurs through the FGF1c receptor (Takashi et al., 2019). Phosphate ion binding to the unliganded FGF1c receptor activated the ERK signalling pathway, leading to *GALNT3* gene upregulation. *GALNT3* encodes an enzyme which *O*-glycosylates FGF23, preventing inactivating proteolysis prior to secretion. These results are compatible with observations that activating FGF1c receptor mutations are associated with hypophosphataemia and increased circulating intact FGF23 (White et al., 2005). Whether both mechanisms by which phosphate ions regulate FGF23 are physiologically operational at dietary phosphorus intakes seen naturally remains to be determined.

3. Acute responses of cats to highly soluble dietary phosphorus

Experimental feeding studies in healthy adult cats have characterised the post-prandial response to diets containing differing phosphorus sources (Coltherd et al., 2019). Feeding a diet where the major phosphorus source was inorganic, in the form of sodium dihydrogen phosphate (SDHP; 3.6 g/Mcal), and the Ca:P ratio was low (0.58:1), led to a marked post-prandial plasma phosphate rise, which peaked at 150 min and remained above baseline for at least the first 6 h (NB: this was the last time-point measured). This was accompanied by a marked decrease in plasma ionised calcium (reaching a stable low point after 90–120 min) and progressive increases in plasma parathyroid hormone (PTH; 2, 3 and 4 h post-prandially), but no change in FGF23. Measurements of PTH were not continued after 4 h and ionised calcium remained reduced from baseline at 6 h when all post-prandial measurements ceased. This contrasts markedly with a diet where all the phosphorus was from organic sources (poultry and bone meal), with a Ca:P ratio of 1.55:1, where a small decrease in plasma phosphate occurred post-prandially. Ionised calcium also decreased, but to a lesser extent (compared to the diet with high inorganic phosphorus), with no change in PTH or FGF23. The post-prandial plasma phosphate decline seen following consumption of organic sources of phosphorus is probably accounted for by slower phosphate ion release, as compared to inorganic sources of phosphorus, together with a post-prandial rise in plasma insulin concentration, which increases cellular phosphate ion uptake (Butterworth et al., 1993; Li et al., 1996).

Coltherd et al. (2019) also demonstrated a dose-dependent effect of dietary inorganic phosphorus on the peak post-prandial plasma phosphate, PTH, and area under curve (AUC) for plasma phosphate over time. Plasma phosphate AUC became positive when inorganic phosphorus, in the form of sodium tripolyphosphate (STPP), was included at 1.5 g/Mcal (Fig. 2). Dietary organic phosphorus had no such dose-dependent effect. Furthermore, SDHP appeared to be more

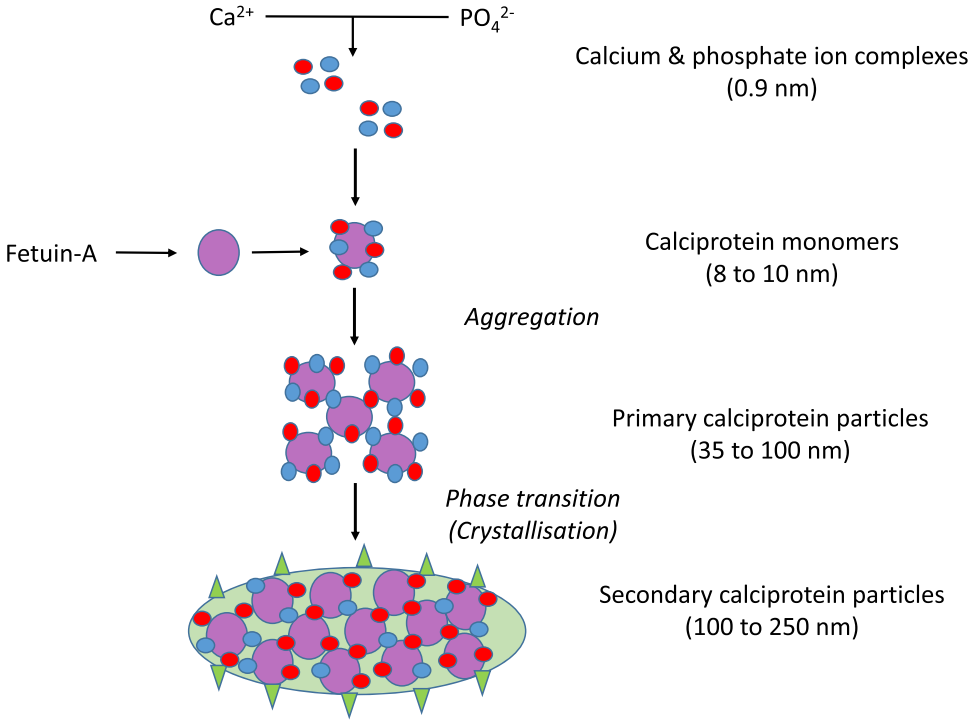

Fig. 1. Schematic representation of the different forms of calciprotein particles. Calcium (Ca^{2+}) and phosphate (PO_4^{2-}) ions form sub-nanometre sized ion complexes. Mineral-binding proteins like fetuin-A bind these ion complexes tightly to form calciprotein monomers. Calciprotein particles are spherical aggregates of these calciprotein monomers. Under certain conditions, primary (amorphous) calciprotein particles transition into secondary (crystalline) calciprotein particles, which are larger and more spiky in shape (the crystalline form in indicated by surface spikes (green) and encapsulating the amorphous form within a more organised structure, indicated by the green oval). For further details on the physical properties of calciprotein particles see Smith et al. (2020).

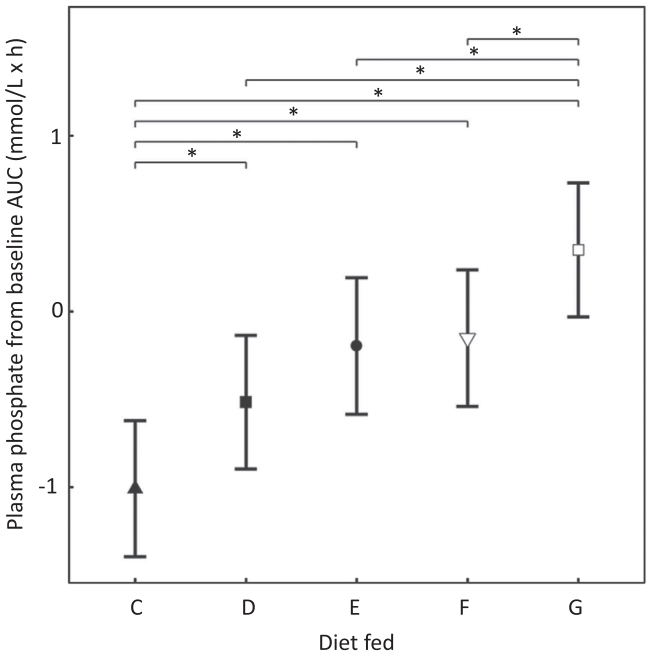

Fig. 2. Effect of dietary inorganic phosphorus (P) content on the post-prandial plasma phosphate concentrations. For each cat ($n = 19$) on each diet ($n = 5$), the plasma phosphate at baseline was subtracted from all post-baseline time points, and the trapezium rule was then used to calculate the area under the curve (AUC) for the 6 h immediately following ingestion of 50% of the daily calorie requirements from the diet specified. Diet C (closed triangle): 1.13 g total P/Mcal; 100% organic P; and Ca:P ratio 1.23:1. Diet D (closed square): 4.02 g total P/Mcal; 0.5 g P from sodium tripolyphosphate (STPP)/Mcal; and Ca:P ratio 1.63:1. Diet E (closed circle): 4.48 g total P/Mcal; 0.75 g P from STPP/Mcal; and Ca:P ratio 1.55:1. Diet F (open triangle): 4.45 g total P/Mcal; 1.0 g P from STPP/Mcal; and Ca:P ratio 1.48:1. Diet G (open square): 4.9 g total P/Mcal; 1.5 g P from STPP/Mcal; and Ca:P ratio 1.64:1. Values are means, with 95% confidence intervals represented by vertical bars. A linear mixed effect model was fitted with AUC as the response. The model fixed effect was diet, and the random effect was diet nested in animal. Asterisks indicate significant differences in mean AUC values between diets ($P < 0{\cdot}05$).
Reproduced with permission from Coltherd et al. (2019).

completely absorbed than STPP as evidenced by the higher AUC for diets containing 1.5 g/Mcal of SHDP when compared with those containing 1.5 g/Mcal of STPP. However, interpretation of these data is further complicated because the diets with different sources of inorganic phosphorus also had slightly different Ca:P ratios with those formulated with STPP tending to have higher Ca:P ratios than those formulated with SDHP. Ca:P ratio also influences the absorption (extent and rate of phosphorus absorption). Coltherd et al. (2019) also showed that diets with the same amounts and sources of inorganic phosphorus, the lower the dietary Ca:P ratio the greater the post-prandial plasma phosphate AUC.

Characterising the post-prandial plasma phosphate response to diets of different composition is important when it comes to determining potential adverse effects on kidney health. Phosphate ions are freely filtered at the glomerulus, and a rise in plasma phosphate and PTH should be accompanied by increased urinary phosphate excretion; however, the methods used by Coltherd et al. (2019) were not sensitive enough to discern differences in urinary phosphate excretion except for the diet containing 3.6 g/Mcal of SDHP. Characterising the post-prandial rise in plasma phosphate can provide very useful information for estimating the proximal tubular phosphate concentration resulting from chronic feeding studies where the same or similar diets were fed (see below).

Finco et al. (1989) compared two diets (cross-over design) of similar phosphorus content but from different sources in a longer-term study. Diet 1's phosphorus content (2.66 g/Mcal; Ca:P 1.57:1) was from organic sources (poultry/fish meal and plant sources) whereas diet 2 had 3.24 g/Mcal of phosphorus, 63.5% (2.06 g/Mcal; Ca:P 1.35:1) of which was from sodium mono and dihydrogen phosphates. The total ration was consumed within 2 h each day. After 20–30 days, blood samples were collected every 2 h post-prandially for 8 h and showed a significant rise in plasma phosphate concentration (from a fasting value of 1.45 mmol/l, peaking at 2.6 mmol/L) when cats were eating diet 2. No such rise was seen when cats consumed diet 1. Feeding diet 2 was associated with a higher proportion of phosphate excreted in urine (34.9 vs. 14.7%) when compared to diet 1. No clinical adverse effects were reported. This study shows that longer-term inorganic phosphorus feeding is associated with post-prandial rises in plasma phosphate, similar to those seen following acute feeding of similar diets, suggesting adaptation to dietary phosphorus composition over 20–30 days, if it occurs, is insufficient to prevent this post-prandial rise. A possible explanation for this could be that inorganic phosphorus absorption is primarily via unregulated paracellular routes.

4. Epidemiological studies showing associations between serum phosphate and CKD development

Increasing evidence suggests that dietary phosphorus intake, particularly of highly available inorganic phosphorus sources, is a risk factor for cardiovascular and kidney disease (Calvo and Uribarri, 2013). It is difficult to estimate inorganic phosphorus consumption, even when detailed dietary questionnaires are completed by human subjects. Many epidemiological studies therefore use serum phosphate as a surrogate. Alternatively, urine phosphate to creatinine ratio or plasma FGF23 have been evaluated as risk factors.

For example, in one ethnically diverse retrospective longitudinal (11 year) cohort study involving 94,989 people enrolled in a vertically integrated health plan, higher serum phosphate levels, but not serum calcium levels, were associated with increased incidence of end stage kidney disease (ESKD) (Sim et al., 2013). After adjustment for other hazards, every 0.16 mmol/L increase in serum phosphate above 1 mmol/L was associated with a 40% higher risk of ESKD. Similarly, Chang and Anderson (2017) showed that risk of incident ESKD increased by 40% for every 0.32 mmol/L increase in serum phosphate above 1.12 mmol/L. This relationship held even for individuals with estimated glomerular filtration rate (eGFR) of $\geq$ 60 mL/min/1.73 m^2 (considered to represent patients without CKD or those with stage 1 or 2 CKD, i.e. with mild to normal kidney function). Blood sample timing will influence the strength of these associations because, in humans, a plasma phosphate circadian rhythm has been noted, the nadir occurring at 11.00 followed by an increase to a plateau at 16.00 with a further secondary peak at 00.30 (Portale et al., 1987). Dietary phosphorus loading changed the timing of these peaks.

Whether such circadian rhythms occur in cats is not known. Epidemiological studies quantifying phosphorus intake and determining whether it is a risk for incident feline CKD are scarce. In a retrospective study of cats diagnosed with CKD, Böswald et al. (2018) were able to estimate previous dietary phosphorus intake in only 16 of 62 cases, but nevertheless demonstrated this was significantly higher than cats presenting to their nutritional clinics with other problems.

5. Predictors of incident azotaemic CKD in cats

CKD is extremely common in aging cats, being the most common cause of death in cats > 5 years of age (O'Neill et al., 2015). In a prospective study involving cats > 9 years old, plasma FGF23, but not phosphate nor PTH, was higher at screening in cats that went on to develop azotaemic CKD within 12 months (Finch et al., 2013). This study did not assess urinary phosphate excretion as a risk factor nor was glomerular filtration rate (GFR) measured. However, cats that became azotaemic had significantly higher plasma creatinine concentrations

than those that remained healthy, suggesting they probably had pre-existing CKD at screening. Similar findings have been published in human diabetic patients with hyperlipidaemia and diabetic retinopathy but without advanced kidney disease (defined as eGFR <30 mL/min/1.73 m^2; Shiizaki et al., 2021) where plasma FGF23 was independently associated with risk of doubling of plasma creatinine or the need for dialysis. Furthermore, human metabolic syndrome patients selected for their kidney function (based on eGFR) being mildly or moderately decreased (eGFR 30–90 mL/min/1.73 m^2; lack of overt proteinuria), where monitoring kidney function is recommended, were followed over three years. In this select group of patients, urine phosphate-to-creatinine ratio at entry was independently associated with their rate of decline of eGFR (Santamaría et al., 2018).

6. Rodent studies demonstrating tubular phosphate damages the nephron

The above epidemiological studies support the hypothesis that increased urinary phosphate excretion resulting from dietary phosphorus overload chronically damages the kidney. Acute phosphate toxicity causes acute tubular necrosis (Ehrenpreis et al., 2011). Rodent phosphorus overload models support this hypothesis and have explored the potential mechanisms. Haut et al. (1980) definitively demonstrated that pathological renal lesions (interstitial oedema, interstitial infiltrate, interstitial fibrosis, tubular atrophy, and tubular dilatation) resulting from feeding of diets supplemented with SDHP preceded the rise in fasting plasma phosphate or creatinine concentrations, challenging the concept that nephrocalcinosis resulted from hyperphosphataemia. Rats with intact kidneys chronically fed (for 10 weeks) a diet containing 2% inorganic phosphorus showed kidney lesions. By using varying degrees of surgical kidney mass reduction and varying the dietary content of SDHP, Haut et al. (1980) were able to demonstrate that kidney lesion severity was highly correlated to the estimated phosphate load per nephron. Vascular calcification was only seen in those rats that developed uraemia. Using a similar rat model, Santamaría et al. (2018) also demonstrated pathological lesions after 3 weeks of high phosphorus diet (2%) feeding, which was exacerbated in those that had undergone unilateral nephrectomy. Tubulointerstitial lesions were present with inflammatory cell infiltrate, centring on proximal tubules where loss of epithelial cell brush border integrity was evident. Tubular cell proliferation in an attempt to repair the damage also occurred. Evidence of renal oxidative stress resulting from high phosphorus diets was inferred based on higher renal glutathione peroxidase activity in the high phosphorus diet rats. An inverse correlation between renal klotho expression and glutathione peroxidase activity suggested that oxidative stress reduced renal klotho production in this model.

Using a different model of salt-sensitive hypertension, Wang et al. (2020) demonstrated that when salt-sensitive rats were fed a high sodium diet (8% NaCl), their urinary phosphate excretion increased without fasting serum phosphate concentrations changing but associated with elevated circulating FGF-23 concentrations. Presumably, high dietary sodium increased intestinal phosphate ion absorption through sodium-dependent transporters despite the fact that the diet contained 0.3% phosphorus, a normal level for rats. After 4 weeks of high salt intake, these rats were hypertensive and albuminuric. Feeding an intestinal phosphorus binding agent, sucroferric oxyhydroxide, had no antihypertensive effect but ameliorated the development of albuminuria and prevented the phosphaturia and the increase in plasma FGF23. The development of renal (tubulointerstitial and glomerular) pathology was also reduced by the administration of sucroferric oxyhydroxide, as was proximal tubular expression of pro-inflammatory mediators, monocyte chemotactic protein-1, and osteopontin. These data support the concept that proximal tubular cell exposure to a higher filtrate phosphate ion concentration invokes a pro-inflammatory response, attracting macrophages into the kidney. Furthermore, renal klotho expression was reduced in this model and this reduction in klotho expression was prevented by sucroferric oxyhydroxide treatment. Wang et al. (2020) speculated that CPPs formed in tubular fluid as tubular phosphate concentration increased.

Shiizaki et al. (2021) provided more definitive evidence that the pro-inflammatory responses induced by dietary phosphorus overload result from the formation of CPPs in proximal tubular fluid and their interaction with Toll-like receptor-4 (TLR-4) expressed in the cortico-medullary junction (CMJ). TLR-4 had been identified as a putative proximal tubular CPP binding site because it is a pattern-recognition receptor, likely to bind CPPs, and is highly expressed in the CMJ. CPPs were labelled by injecting mice with a fluorescent bisphosphonate, which binds to crystalline calcium phosphate. Mice that had been fed a high phosphorus diet (2% inorganic phosphorus) for 8–9 days showed fluorescent signals from the CMJ where the last proximal tubule segment (S3) is located. Tubule damage, inflammation, and fibrosis centred on the CMJ (Fig. 3) with associated up-regulation of multiple genes encoding for markers of tubular damage or mediators of pathology were also noted. Acidifying the urine (via addition of ammonium chloride to drinking water) or knocking out TLR-4 expression on the apical proximal tubular cell surface (renal conditional *Tlr-4* gene knockouts) reduced the fluorescent signal from the CMJ. Histological and molecular-pathology was also much reduced in the TLR-4 knockout mice, despite phosphate excretion per tubule being the same as in wild-type mice. Theoretical calculations of the 24 h average phosphate concentration in the S3 segment that represents the threshold for fibrosis and nephron loss was > 3 mmol/L.

These three papers (Santamaría et al., 2018; Wang et al., 2020; Shiizaki et al., 2021) strongly suggest that phosphate toxicity is initiated when the phosphate concentration in the S3 segment rises above a threshold concentration such that CPPs are formed. The precise nature (size and shape) of the CPPs which form in the proximal tubule remains to be determined. All three papers contain cell culture studies involving either human (HK2 and HEK293) or rat (NRK-52 E) kidney cell lines that recapitulate these effects in vitro. Similar threshold concentrations for the oxidative stress and pro-inflammatory effects of phosphate ions (2.6–3.3 mmol/L; Santamaría et al., 2018) were documented with effects becoming evident after 6–24 h of incubation. Detailed sub-cellular analysis of CPP interaction with human kidney cells (HK2) has since demonstrated that CPPs accumulate in late endosomes/lysosomes, resulting in autophagocytic flux blockade and oxidative stress (Kunishige et al., 2020).

7. Evidence that excessive inorganic phosphorus intake damages the cat kidney

Ross et al. (1982) were the first to show that dietary phosphorus restriction reduced the interstitial fibrosis, inflammation, and mineralisation, albeit in a remnant kidney model of chronic kidney disease.

In 1995, Pastoor et al., undertook a 4-period cross-over study to determine the minimum phosphorus requirement of young healthy adult neutered female cats. Four diets were tested that varied 6-fold in total and 9.6-fold in inorganic phosphorus content. All diets had more than 50% of their phosphorus content coming from SDHP (Appendix A: Supplementary material, Table S1). The Ca:P ratio was inversely proportional to the phosphorus content varying from 0.4 to 2.3–1. Each feeding period lasted 28 days, during the last 7 days of which cats were individually housed and balance studies undertaken. Phosphorus bioavailability and urinary phosphate excretion increased with dietary SDHP content and phosphorus balance was achieved on all diets. Feeding the highest phosphorus diet (Diet 4; 3.35 g/Mcal inorganic phosphorus) was associated with detrimental effects (reduced food intake, 28.5% reduction in GFR, raised serum alkaline phosphatase activity, and fasting hypophosphataemia). No carry-over effect of Diet 4 into subsequent periods was reported suggesting that the effect of Diet 4 on GFR was reversible. Unfortunately, no phosphaturic hormones were measured in this study.

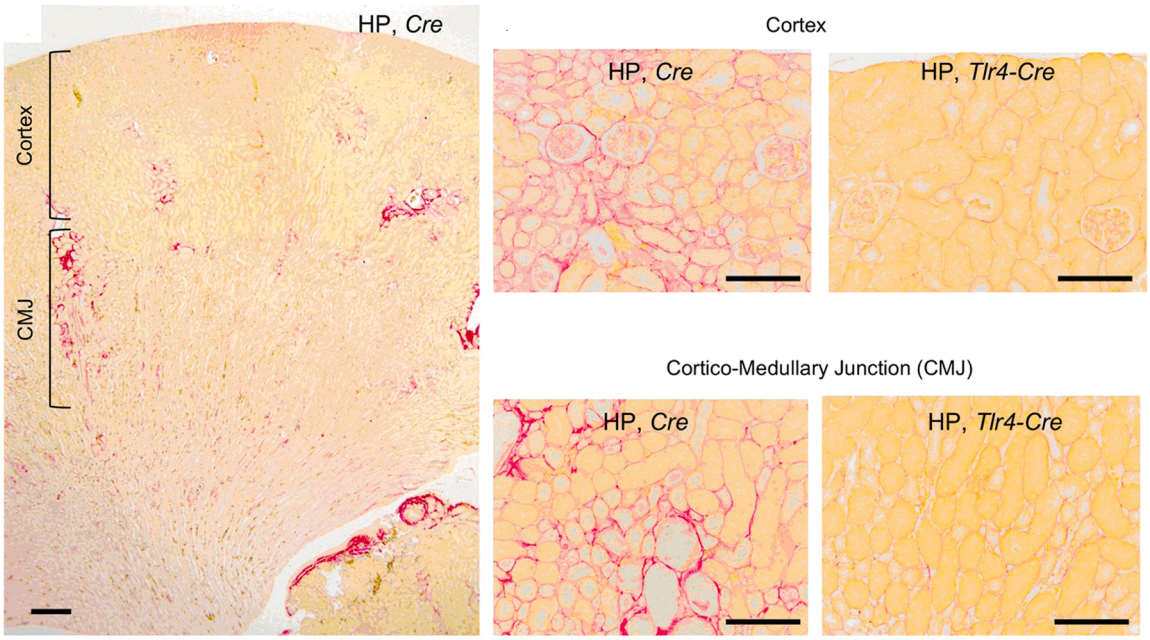

Fig. 3. Feeding of a high phosphorus diet induces renal interstitial fibrosis in mice, via binding of calcium phosphate particles to Toll-like receptor-4 (TLR-4) (Shiizaki et al., 2021). Mice lacking the *Tlr4* gene in renal tubular cells (*Tlr4-Cre*) and control mice (*C re* ; carrying the *C re* transgene alone) at 4 weeks of age were placed on a high-phosphorus diet containing 2.0% inorganic phosphorus (HP) for 4 weeks. Picrosirius red staining of the kidney sections detected patchy red areas of interstitial fibrosis in the cortex and the cortico-medullary junction (CMJ) in the control mice (HP, *C re*) but not in the mice lacking the *Tlr4* gene in renal tubular cells (HP, *Tlr4-Cre*). Scale bars: 100 μm.
Reproduced with permission from Shiizaki et al. (2021).

Dobenecker et al. (2018) undertook a cross-over diet trial to determine whether feeding high levels of highly available inorganic phosphorus was toxic to kidneys of healthy adult cats. They compared two home-made diets fed for 29 days with a 12-month washout period. The composition of the diets is shown in Appendix A (Supplementary material, Table S2). The control diet provided 1.6-times the National Research Council (NRC) recommended maintenance phosphorus requirements. The test diet had 3-times more phosphorus than the control diet, including 2 g/Mcal inorganic phosphorus salts (calcium and sodium monophosphates) whereas all the phosphorus in the control diet was from organic sources. Other major differences (control vs. test) were the Ca:P ratio (1.3 vs 0.4:1) and sodium content (test diet 4.3-times control). Detrimental effects of test diet feeding on kidney function included a lower GFR (23.4% lower than when eating the control diet), and evidence of proximal tubular stress (both albuminuria and glucosuria were seen in nine out of 13 cats on the test diet but in none on the control diet). Nevertheless, cats produced concentrated urine on both control and test diets (urine specific gravity: 1.046 ± 0.006 vs. 1.062 ± 0.008). Phosphorus was much more bioavailable from the test diet leading to 7-times more urinary phosphate excretion when the test diet was consumed.

Alexander et al. (2019) undertook two parallel-group design studies. The first involved 48 healthy adult cats block-randomised (based on body weight, age, and energy intake) to receive either a control diet (providing 1.3 g/Mcal of phosphorus all from organic sources; Ca:P ratio 1.3:1) or a test diet (providing 4.78 g/Mcal of phosphorus, 3.6 g/Mcal of which was supplied from SDHP; Ca:P ratio 0.59:1), similar to Diet 4 in the Pastoor et al. (1995) study (Appendix A: Supplementary material, Table S3). All cats had been acclimatised to the control diet for 20 weeks prior to study commencement and to consuming 50% their daily ration every 12 h, a feeding regimen adopted throughout the study. Cats fed the test diet showed adverse health effects (inappetence, vomiting) from week-4 and the trial was stopped at week-6 following review of biochemical data. Cats fed the test diet had lower GFR, lower fasting plasma phosphate concentrations, and higher urinary albumin excretion than those fed the control diet. An expert diagnostic imager identified renal structural changes on ultrasound (increases in renal echogenicity) of varying severity in 22 of the 24 (92%) cats fed the test diet cf. one of the 24 cats fed the control diet. Plasma FGF23 and PTH were 5- and 2-fold higher, respectively, after 4 weeks on the test diet when compared to the cats fed the control diet.

The second study by Alexander et al. (2019) enrolled 50 healthy adult cats and block-randomised them to receive either a control or test diet (composition shown in Appendix A: Supplementary material, Table S4). The control diet was similar in composition to study 1 and had been fed as the baseline diet for all cats for 10 weeks prior to study commencement. The test diet had 1.50 g/Mcal of inorganic phosphorus (as SDHP), constituting 42% of the total phosphorus. This study ran for 28 weeks. Blood and urine samples were collected, GFR measured, and mineral balance studies undertaken every 4–8 weeks. Four cats dropped out of the study for behavioural reasons and three test group cats were removed at week-21 because of concerns over increasing plasma creatinine concentrations. Within 2 weeks, the test group's plasma FGF23 was elevated (3-fold compared to baseline). Test cats had 2.7-fold higher urinary phosphate excretion than control cats after 4 weeks. After 4 weeks, the test group's urinary albumin was significantly higher than baseline (and control) and remained so for the study's duration. Nine test group and no control group cats had renal structural changes on ultrasound imaging. Nephroliths were found in 60% and 27% of test and control cats respectively.

Taken together, these four studies demonstrate that feeding highly bioavailable phosphorus sources to adult cats results in proximal tubular stress (as indicated by presence of albuminuria with or without glucosuria), structural changes to the kidney, and functional reduction in filtration. None of these studies undertook renal histopathology so it is not possible to compare phosphate-induced renal pathology between cats and rodents. Because the two experimental test diets from Alexander et al. (2019) were also fed in acute feeding studies (Coltherd et al., 2019), it is possible to calculate the theoretical proximal tubular (S3 segment) concentrations of phosphate in the post-prandial and fasting

Table 2
Estimated (calculated) changes in phosphate concentration along the proximal convoluted tubule associated with different dietary phosphorus intake by cats.

Reference study	Equivalent diet[a] (inorganic phosphorus content; Ca:P ratio)	Change in tubular phosphate concentration along the proximal tubule (S1 to S3 segments; mmol/L)[b]					Fractional excretion of phosphate (%)	
		Control		Test			Control	Test
		S1	S3	PPr/Fa	S1	S3		
Alexander et al. (2019) (study 1)	Diet A 3.6 g SDHP/Mcal Ca:P ratio 0.6:1	1.3 (1.2–1.4)	0.54 (0.43–0.67)	PPr (8 h) Fa (16 h)	1.98 (1.7–2.3) 1.46[c] (1.3–1.7)	4.95 (4.25–5.75) 3.65 (3.25–4.25)	12.5 (10.84–14.41)	75% (maximal)
Alexander et al. (2019) (study 2)	Diet K 1.5 g SDHP/Mcal Ca:P ratio 0.9:1	1.35 (1.25–1.45)	0.92 (0.72–1.12)	PPr (10 h) Fa (14 h)	1.57 (1.44–1.7) 1.38[c] (1.3–1.5)	3.95 (3.60–4.25) 3.45 (3.25–3.75)	20.5 (17.25–23.27)	75% (maximal)
Coltherd et al. (2021) (Moderate Total Phosphorus)	Diet F 1.0 g STPP/Mcal Ca:P ratio 1.48:1	1.40 (1.2–1.6)	0.82 (0.61–1.07)	PPr (2 h) Fa (22 h)	1.50 (1.38–1.63) 1.25[c] (1.15–1.35)	2.20 (1.93–2.54) 1.83 (1.61–2.11)	17.59 (15.15–20.03)	44 (42.0–46.8)
Coltherd et al. (2021) (High Total Phosphorus)	Diet F 1.0 g STPP/Mcal Ca:P ratio 1.48:1	1.40 (1.2–1.6)	0.82 (0.61–1.07)	PPr (2 h) Fa (22 h)	1.50 (1.38–1.63) 1.35[c] (1.25–1.45)	1.59 (1.34–1.86) 1.42 (1.22–1.65)	17.59 (15.15–20.03)	31.75 (29.31–34.19)

Fa, fasting; PPr, post-prandial; SDHP, sodium dihydrogen phosphate; STPP, sodium tripolyphosphate.

[a] Equivalent diet fed acutely in Coltherd et al. (2019) on which the post-prandial changes in plasma phosphate are based.

[b] For each long-term feeding study the nearest equivalent diet from the acute feeding study (Coltherd et al., 2019) has been used to predict the change in plasma phosphate concentration post-prandially over time and the time spent at the high post-prandial level has been estimated from the kinetic graphs of acute feeding.

[c] The fasting plasma phosphate concentration is taken from the long-term feeding study (mean and 95% confidence limits) and, where plasma phosphate increased in the acute kinetic studies (Diets A and K; Alexander et al., 2919; studies 1 and 2) to account for the change in plasma phosphate that occurs after the peak post-prandial concentration starts to fall, a phosphate concentration mid-way between the post-prandial peak and the fasting concentration has been used. For example, for Alexander et al. (2019) study 1, the mean fasting (12 h) plasma phosphate was 0.95 mmol/L (range 0.85–1.05 mmol/L), the mean peak post-prandial phosphate was 1.98 mmol/L (range 1.7–2.3 mmol/L), thus an average plasma phosphate concentration of 1.46 mmol/L (range 1.3–1.7 mmol/L) has been used to calculate the change in phosphate concentration along the length of the proximal tubule during the fasting phases (6–12 h and 18–24 h). The following assumptions have been used to determine the tubular phosphate concentration: at S1 (filtered = plasma concentration) and the concentration at the end of the proximal tubule (S3 segment) is calculated by assuming 70% of the water is reabsorbed. The percentage of filtered phosphate excreted is either measured for control diets in all studies (Alexander et al., 2019, studies 1 and 2; Coltherd et al., 2021) or estimated at 75% in the test diets (Alexander et al., 2019, studies 1 and 2, where plasma phosphate changed significantly post-prandially, such that the measured fractional excretion of phosphate in the paper is inaccurate). This estimate is based on the fractional excretion of phosphate in cats with CKD being maximal at 75% (unpublished data from Elliott et al., 2003). For the test diets in Coltherd et al. (2021), the measured fractional excretion of phosphate reported in the paper resulting from the test diet feeding at week 27 have been used as very small changes in plasma phosphate concentration occurred so the estimated fractional excretion of phosphate using fasting plasma phosphate is likely to be accurate.

states, assuming little or no adaptation occurs to more chronic phosphorus feeding (Table 2). The assumptions made in calculating the change in concentration along the proximal tubule length are explained in the table's legend and are based on assumptions verified by Shiizaki et al. (2021) and their theoretical basis appears to be valid for the cat (Friedman and Roch-Ramel, 1977). In the studies by Alexander et al. (2019), for cats fed test diets where proximal tubular stress was noted, proximal tubular phosphate concentration exceeded 3.25 mmol/L, even in the fasted state. Based on the work of Shiizaki et al. (2021), it seems likely that CPPs form in the filtrate at this phosphate concentration and continuously overload the tubular cells' endolysosomes. Two experimental conditions are likely to have magnified the experimental diets' effects in the studies by Alexander et al. (2019). First, cats were acclimatised to a low phosphorus diet with no added inorganic phosphorus for 20 or 10 weeks, presumably maximally upregulating intestinal phosphate-absorbing systems in advance of a diet change. Second, and a condition that applies to all four studies discussed above, cats were meal fed rather than offered food ad libitum. Given the option, most cats would eat small multiple meals per day (Parker et al., 2019) which would influence the peak plasma phosphate concentration resulting from a particular diet.

8. What is a safe level of inorganic phosphorus to include in a prepared diet?

This is an important question as there are a number of technical and nutritional reasons why inorganic salts of phosphorus are added to prepared pet foods. Coltherd et al. (2021) addressed this question by feeding two test diets over 30 weeks to healthy adult cats and comparing to a control diet, similar in composition to those used by Alexander et al. (2019); (Appendix A: Supplementary material, Table S5). Both test diets had 1 g STPP /Mcal of as added inorganic phosphorus. One was described as moderate (total phosphorus 4 g/Mcal; Ca:P ratio 1.04:1) and the other as high (5 g/Mcal total phosphorus; Ca:P ratio 1.27:1) in phosphorus. Although urinary phosphate excretion was higher in cats fed these test diets, no indications of proximal tubular stress were evident (as determined by the absence of albuminuria or glucosuria). FGF23 was mildly elevated in the moderate phosphorus diet group from 2 weeks onwards, being on average 46% higher than the control diet group across the trial (range of group median values based on 7 sampling points over the 28-week feeding period: 108–141 pg/mL vs. 156–217 pg/mL for the control and moderate phosphorus diet groups respectively), with higher levels being associated with increased fractional excretion of phosphate as expected. It seems likely that this reflects a physiological response to increased phosphorus intake. No more than 20% of cats fed the moderate phosphorus test diet had FGF23 $>$ 300 pg/mL at any time point which concurs with our findings when screening normal healthy older cats fed a variety of commercially available adult cat foods (Sargent et al., 2019).

Coltherd et al. (2021) undertook detailed renal (GFR measurement, renal imaging), bone (dual-energy X-ray absorptiometry, bone marker monitoring), and general health monitoring and concluded that neither test diet produced detectable adverse health effects after 30 weeks. The authors concluded that 1 g STPP/Mcal could be safely included in adult cat diets. A 30-week feeding study represents $<$ 4% of a cat's lifespan yet it is unrealistic to expect that longer well controlled studies will be conducted. Indeed, 6 months is the recommended length of chronic target animal species safety studies for drug development [1] (EMA 2008). In the acute kinetic studies (Coltherd et al., 2019), the diet fed with 1 g/Mcal STPP (Diet K) had a post-prandial plasma phosphate AUC of 0 and gave only a transient small increase in plasma phosphate. Diet F was the closest in composition to the high and moderate phosphorus diets used in Coltherd et al. (2021). Assuming an equivalent post-prandial plasma phosphate peak to Diet F occurs chronically for both the moderate and high phosphorus diets, as was seen with the acute feeding of Diet F (Coltherd et al., 2019), S3 segment proximal tubular phosphate concentrations are predicted to have only transiently exceeded 2 mmol/L in the majority of cats fed the moderate phosphorus diet and are predicted not to have exceeded this concentration in any cat fed the high phosphorus diet. These theoretical calculations support the conclusions that these diets are likely to be safe in healthy adult cats.

More prolonged feeding of diets containing inorganic phosphorus at 1 g/Mcal has been undertaken without evidence of detrimental health effects. Reynolds et al. (2013) fed two prepared diets each containing inorganic phosphorus at 1 g/Mcal (0.5 g potassium monophosphate and 0.5 g pyrophosphate) with a total phosphorus content of 2.18 g/Mcal and a Ca:P ratio of 0.85:1. The diets differed in sodium content (1.02 vs. 3.26 g/Mcal) and the goal of the study was to determine the long-term effects of feeding a high sodium diet. Twenty healthy adult senior cats were involved in this 2-year study, 75% of which were $\geq$ 10.9 years old. Sixteen completed the study, four being withdrawn due to cancer (two cases), diabetes mellitus (one case), and one dying of unknown cause. No evidence of deteriorating renal function (based on serial GFR measurements), structural renal changes (based on detailed ultrasound examinations) or renal tubular stress (based on urine albumin measurements) was identified. This contrasts with UK pet cats $\geq$ 10 years old followed for 12 months after health screening where 30.5% developed azotaemic CKD (Jepson et al., 2009).

9. What factors might influence the safety of manufactured diets containing 1 g of inorganic phosphorus/Mcal for healthy adult cats?

It is important to recognise that the rate and extent of phosphorus absorption from the GI tract can be influenced by multiple factors, many of which we lack detailed information on in the cat. The current literature and knowledge gaps in this area are highlighted in a recent review (Laflamme et al., 2020). Factors shown to influence the peak post-prandial serum phosphate concentration (and therefore the starting phosphate concentration in the proximal tubule) include the source of inorganic phosphorus, the Ca:P ratio, and the sodium content of the food. In addition, the proportion of the daily ration consumed at each meal (i.e. whether once daily, twice daily, or ad lib feeding) will also affect the peak post-prandial serum phosphate concentration. The impact of these has been discussed earlier and would need to be taken into account when formulating a diet including inorganic sources of phosphorus (see Coltherd et al., 2019 where acute feeding studies demonstrate some of these differences). Other dietary factors include the amount of other divalent cations in the food (e.g. magnesium) and the source and amount of dietary fibre, all of which may influence the rate and reduce the extent of inorganic phosphorus absorption. As multiple factors in a formulated diet can influence the rate and extent of phosphorus absorption, which appears to be critical to its effects on renal health, the recommendation that diets contain inorganic phosphorus $\leq$ 1 g/Mcal and have a Ca:P ratio of $\geq$ 1:1, which is supported by evidence in the studies reviewed above, should be viewed as a starting point when formulating a prepared diet. Given the reviewed studies have been done primarily with twice daily feeding, this practice or ad lib feeding should be recommended for similarly formulated diets. The impact of once daily feeding on phosphorus absorption and the resulting exposure of proximal tubular cells to phosphate remains to be determined. Finally, as nephrons are lost in the early stages of CKD, the amount of phosphate excreted per nephron increases via adaptive increases in serum FGF23, changes which have been documented to occur in pre-azotaemic CKD (Finch et al., 2013). Although detailed studies

[1] See: European Medicines Agency. EMEA/CVMP/VICH/393388/2006. Guideline on target animal safety for veterinary pharmaceutical products. London September 2008. https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl43-target-animal-safety-veterinary-pharmaceutical-products-step-7_en.pdf (Accessed 14 December 2021).

have not been undertaken of the benefits of dietary phosphorus restriction at this stage of CKD, it would seem prudent to further limit the intake of inorganic phosphorus if early CKD has been diagnosed or is suspected.

10. Conclusions

Knowledge of phosphorus homeostasis has increased in recent years with the discovery of klotho, FGF23, and CPPs. Rapidly absorbed inorganic forms of phosphorus added to the diet are potentially harmful to the kidney if the entry rate of phosphorus into the body exceeds the body's ability to dispose of it safely. Homeostatic mechanisms that inhibit proximal tubular phosphate ion reabsorption, in an attempt to increase urinary phosphate excretion, appear to be associated with structural changes in cat kidneys if they lead to proximal tubular (S3 segment) fluid phosphate concentrations exceeding 3.25 mmol/L for significant periods. This mirrors the experimental in vivo threshold in rodents for the development of renal fibrosis and nephron loss and is compatible with available cell culture data. Diets containing levels of inorganic phosphorus, which lead to proximal tubular S3 segment fluid concentrations that are well below 3.25 mmol/L, were not associated with detectable detrimental health effects. This suggests that the addition of forms of inorganic phosphorus at or below 1 g/Mcal can be safe for healthy adult cats provided attention is paid to the form of inorganic phosphorus (data available primarily relate to sodium tripolyphosphate and potassium monophosphate/pyrophosphate) and other dietary factors that might influence phosphorus bioavailability.

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Conflict of interest statement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2022.105842.

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