Dietary Phosphorus in Chronic Kidney Disease: Effects of Amount, Source and Bioaccessibility on Intestinal Absorption and Health Outcomes

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Kendal Mayer Burstad

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Advisor: Kathleen Hill Gallant, PhD, RD
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Dedication

This dissertation is dedicated to all of those who have supported, guided, and mentored me throughout my graduate career. I could not have done it without you.

Abstract

Phosphorus restriction is a key component to dietary recommendations for patients with chronic kidney disease (CKD) to aid in the prevention of CKD-mineral bone disorder (CKD-MBD). However, this is challenging and burdensome to follow, leading to bouts of non-adherence. How these bouts of non-adherence affect intestinal phosphorus absorption remains unclear. In addition, other approaches to manage dietary phosphorus intake are of growing interest such as the incorporation of plant-based protein. How these new dietary approaches to manage phosphorus intake affect intestinal phosphorus absorption or other health outcomes in CKD must be determined. In this dissertation, we aimed to evaluate how dietary phosphorus amount, source, and bioaccessibility affect intestinal phosphorus absorption and health outcomes in CKD.

We first sought to determine the effect of acute high dietary phosphorus intake following acclimation to a low phosphorus diet on intestinal fractional phosphorus absorption using an *in vivo* oral gavage technique in a rodent model of CKD. Despite finding no difference in intestinal fractional phosphorus absorption between groups, plasma phosphorus, fibroblast growth factor-23, and parathyroid hormone were all significantly higher in rats in the low to high phosphorus and high phosphorus groups compared to the low phosphorus group. These findings support continued efforts to reduce phosphorus intake in patients with CKD.

We then aimed to determine phosphorus bioaccessibility of emerging plant-based protein products as a new approach to manage dietary phosphorus intake. We found that average phosphorus bioaccessibility ranged from ~32% in pulse-based beef to ~100% in pulse-based milk. Despite this large range in percent bioaccessible phosphorus, most of the plant-based protein products evaluated had lower phosphorus bioaccessibility in mg per 100g serving

compared with animal-based protein products. However, how this translates *in vivo* is still unknown.

Additionally, we undertook a systematic review to summarize the available clinical trial evidence for the effect of plant-based protein on kidney function and MBD outcomes in adults with stage 3-5 CKD not on dialysis. Overall, results for both kidney function and CKD-MBD outcomes were heterogenous and most studies were of suboptimal methodological quality. Of the included studies, a subset of five investigated a change in protein source only (i.e., animal vs plant). No change in kidney function was reported in four studies, while one study, of longer duration, reported a decrease. Further, of the CKD-MBD outcomes only one short term study reported lower serum phosphorus following a vegetarian diet.

While our results from the study of intestinal phosphorus absorption in rodents support continued efforts to reduce phosphorus intake in patients with CKD, it is evident that other approaches to help manage phosphorus intake in this population are required. Our findings for phosphorus bioaccessibility indicate that emerging plant-based proteins may be suitable options for patients with CKD as they offer lower phosphorus bioaccessibility compared with animal products. However, our systematic review results show that sparse data with heterogenous results are available for the effect of plant-based protein compared with animal protein on kidney function and CKD-MBD outcomes in adults with stage 3-5 CKD not on dialysis. Therefore, more research must be conducted to determine the health effects of plant-based protein consumption to manage phosphorus intake in patients with CKD.

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List of Abbreviations

GFR – Glomerular Filtration Rate

eGFR – Estimated Glomerular Filtration Rate

CKD – Chronic Kidney Disease

CVD – Cardiovascular Disease

CKD-MBD – CKD-Mineral and Bone Disorder

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

FGF-23 – Fibroblast Growth Factor-23

PTH – Parathyroid Hormone

$25,OHD_3$ – 25-Hydroxyvitamin D

$1,25OH_2D_3$ – 1,25 Dihydroxyvitamin D

NaPi-2b – Type II Sodium Dependent Phosphate Transporter 2b

NaPi-2a – Type II Sodium Dependent Phosphate Transporter 2a

NaPi-2c – Type II Sodium Dependent Phosphate Transporter 2c

PiT-1 – Type III Sodium Dependent Phosphate Transporter 1

PiT-2 – Type III Sodium Dependent Phosphate Transporter 2

KO – Knockout

NHE3 – Sodium Hydrogen Exchanger 3 Inhibitors

BBMV – Brush Border Membrane Vesicles

VDR – Vitamin D Receptor

PTHR1 – Parathyroid Hormone 1 Receptor

CaSR – Calcium Sensing Receptor

FGFR1 – Fibroblast Growth Factor 1

SHPT – Secondary Hyperparathyroidism

KDOQI – Kidney Disease Outcomes Quality Initiative

KDIGO – Kidney Disease Improving Global Outcomes

RCT – Randomized Controlled Trial

NHANES WWEIA – National Health and Nutrition Examination Survey – What We Eat in America

RDA – Recommended Dietary Allowances

UL – Tolerable Upper Intake Level

FDA – Food and Drug Administration

NDSR – Nutrient Data Systems for Research

LDL – Low-Density Lipoprotein

Non-HDL – Non-High-Density Lipoprotein

I.V. – Intravenous

LP – Low Phosphorus Diet

HP – High Phosphorus Diet

LPHP – Low Phosphorus Followed by Acute High Phosphorus Diet

ANCOVA – Analysis of Covariance

NSAID – Non-Steroidal Anti-Inflammatory Drug

^{33}P – ^{33}P-Orthophosphorus Acid

PBS – Phosphate Buffered Saline

Na_2HPO_4 – Disodium Phosphate

AUC – Area Under the Curve

CO_2 – Carbon Dioxide

NaCl – Sodium Chloride

mRNA – Messenger Ribonucleic Acid

PCR – Polymerase Chain Reaction

RPLP0 – Ribosomal Protein, Large, P0

BUN – Blood Urea Nitrogen

Ca – Calcium

ELISA – Enzyme-Linked Immunosorbent Assay

EIA – Enzyme Immunoassay

SAS – Statistical Analysis Software

SDR – Studentized Deleted Residual

SD – Standard Deviation

MP-AES – Microwave Plasma Atomic Emission Spectrometer

INFOGEST – International Network of Excellence on the Fate of Food in the Gastrointestinal Tract

$CaCl_2$ – Calcium Chloride

OPM – Oscillations Per Minute

MWCO – Molecular Weight Cut Off

TMAO – Trimethylamine-N-Oxide

LPD – Low Protein Diet

VLPD – Very Low Protein Diet

ESKD – End-Stage Kidney Disease

MeSH – Medical Subject Headings

ROB-2 – Risk of Bias-2 Tool

KA – Ketoanalogue

CrCl – Creatinine Clearance

Chapter 1: Introduction

Kidney Physiology in Health and Disease

The kidney is a vital organ for the removal of waste products in the blood as it filters ~200 liters of blood per day.[1] The kidney also plays important roles in acid-base balance, production of erythropoietin (critical for the stimulation of red blood cell production), conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, and production of renin for blood pressure regulation.[1–6] Kidney function is determined by glomerular filtration rate (GFR), the rate of fluid and solute filtered by the kidney per minute.[1] A decline in GFR indicates suboptimal functioning of the kidney and can lead to chronic kidney disease (CKD), defined as the presence of kidney damage or reduced kidney function for >3 months.[7,8]

CKD is a growing public health concern affecting ~37 million U.S. adults and is the eighth leading cause of death in the United States.[7,9] The two major risk factors influencing progressive decline in kidney function include hypertension and type II diabetes mellitus.[10,11] Other risk factors include age, sex, dyslipidemia, smoking, obesity, and history of cardiovascular disease (CVD).[8,11,12] Loss of kidney function is characterized into five stages; in stage one, GFR is normal or even higher than normal (≥ 90 mL/min/1.73m^2) when there is only mild kidney damage, whereas in stage five GFR is severely decreased (<15 mL/min/1.73m^2) and renal replacement therapy through dialysis or transplant is needed for survival.[8]

Kidney function decline is associated with a variety of health complications. In fact, those with an eGFR of 15-30 mL/min/1.73m^2 and an albumin-to-creatinine ratio of >300 mg/g were found to have an 8.1-fold greater risk of CVD mortality.[13] Additionally, Thompson et al., show that CVD is a primary cause of death in patients with an eGFR <60 mL/min/1.73m^2.[14] Other potential health complications include anemia, anorexia, fatigue, infection, disturbances in acid-base balance, CKD-mineral bone disorder (CKD-MBD), and decreased quality of life.[15,16] One major contributor to these health complications is the dysregulation of phosphorus metabolism that occurs as kidney function declines.[16–18]

Roles and Regulation of Phosphorus Metabolism

Phosphorus, an abundant mineral in the body, is an essential nutrient required for proper function of multiple body processes. Phosphorus plays a major role in cell membrane structure, energy metabolism, enzymatic activation, and is a key component of bones, teeth DNA and RNA.[19,20] Eighty-five percent of phosphorus is found in bones and teeth and 15% is distributed in soft tissues and blood.[20,21] The normal range for serum phosphorus is 3.4-4.5 mg/dL.[20,21] Phosphorus is consumed through many foods and is present in both organic and inorganic forms. Organic phosphorus is found in protein rich foods such as meat, poultry, fish, dairy products, whole grains, nuts, pulses etc. and must be hydrolyzed to inorganic phosphorus prior to absorption.[22] Phosphate salts make up inorganic phosphorus and are not bound to protein, thus are more readily

absorbed. Inorganic phosphorus is found in foods such as beverages, cereals, frozen meals, and deli meats.[22]

Phosphorus homeostasis is maintained through a multi-tissue axis involving the bone, intestine, kidney and parathyroid gland.[18] The major hormones involved include fibroblast growth factor-23 (FGF-23), parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D ($1,25OH_2D_3$) (**Figure 1.1**).

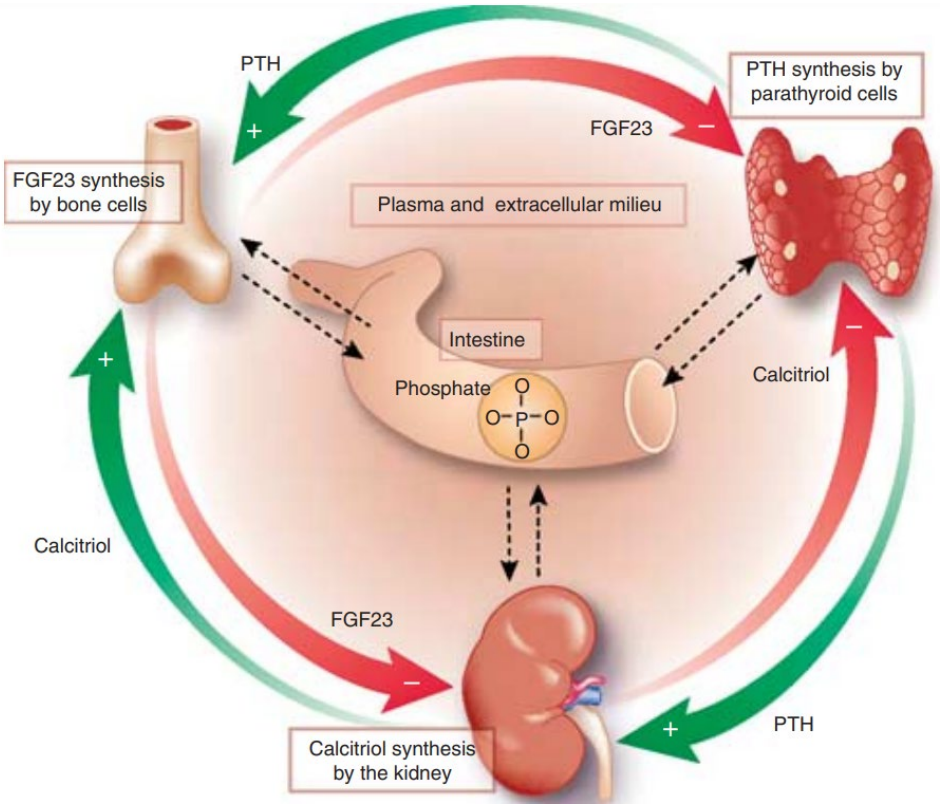

Figure 1.1. Maintenance of phosphorus homeostasis through a multi-tissue axis

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Intestinal Phosphorus Absorption

Intestinal phosphorus absorption occurs via two major routes, the transcellular route or active transport and the paracellular route or passive transport.[24,25] Active transport is mediated by sodium dependent phosphate cotransporters which allow for the absorption of phosphorus through enterocytes whereas passive transport is mediated by an electrochemical gradient in which phosphorus passes between the tight junctions of enterocytes (**Figure 1.2**).[25,26] These differences in intestinal phosphorus absorption were discovered by McHardy and Parsons[27] as they observed a linear increase in intestinal phosphorus absorption with increasing luminal phosphorus concentrations in rats. Similar results were observed in the jejunum and ileum of humans following a triple lumen perfusion absorption experiment.[28,29] A majority of phosphorus absorption occurs in the small intestine, but some may also be absorbed in the large intestine.[30–32] However, the extent to which phosphorus is absorbed in the large intestine is still unclear, but the longer residence time (relative to the small intestine) presents the possibility of this contributing substantially to overall phosphorus absorption.

Known transporters involved in phosphorus absorption include type II sodium dependent phosphate transporter 2b (Napi-2b, *SLC34A2*) and type III sodium dependent phosphate transporter 1 and 2 (PiT1, *SLC20A1* and PiT2, *SLC20A2*). The majority of transcellular absorption is understood to be driven by Napi-2b and occurs to a lesser extent by PiT1 and PiT2.[24,33–36] This was

demonstrated through the study of mouse models using NaPi-2b knockouts (KO). It was observed that NaPi-2b KO mice absorbed ~50% less phosphorus than controls after receiving an oral gavage phosphate bolus.[37] This has also been demonstrated in uremic mice.[38] These data suggest that NaPi-2b is indeed the predominant phosphate transporter, at least in mice. Phosphate transporters NaPi-2b and Pit-1 are localized to the proximal intestine of rats (i.e., duodenum and jejunum).[39] However, transporter expression and absorptive capacity differs across species.[27–29,40,41] Rat models were found to be most similar to humans as both humans and rats have similar phosphate transporter expression along the intestine and have greater absorptive capacity in the duodenum and jejunum compared with the ileum.[25,27,28,30,39]

Luminal phosphorus concentration and $1,25OH_2D_3$ are two major factors that impact intestinal phosphorus absorption.[34,39,41–48] Other factors that affect total phosphorus absorption include nicotinamide,[37,49–51] phosphorus binders,[52–54] and sodium hydrogen exchanger 3 inhibitors (NHE3).[24,55] Here, we will focus on luminal phosphorus concentration and $1,25OH_2D_3$. As mentioned above, there is a linear relationship between luminal phosphorus concentration and phosphorus absorption. When luminal phosphorus concentration is low, transcellular absorption predominates, increasing absorption efficiency whereas when luminal phosphorus concentration is high, phosphate transporters become saturated and a majority of phosphorus is absorbed paracellularly.[25,39] *In vitro* brush border membrane vesicle (BBMV) uptake experiments demonstrate greater phosphorus

uptake in rodents on a low phosphorus diet (0.1% or 0.09%) compared with a high phosphorus diet (1.1 or 1.2%).[34,39] As expected, this was also accompanied by higher NaPi-2b protein expression.[34]

The upregulation of phosphate transporter expression and increased phosphorus absorption efficiency observed on a low phosphorus diet may adversely affect phosphorus absorption if an acute high phosphorus load is consumed (as may be seen during a bout of non-adherence to low phosphorus diet recommendations in CKD). This was observed by Giral et al.[39] in which healthy male rats acutely switched to a high phosphorus diet following consumption of a low phosphorus diet had even greater BBMV phosphorus uptake and 3-fold higher serum phosphorus than rats that continued on the low phosphorus diet. These data suggest that deviations from a low phosphorus diet could lead to a maladaptive response of increased phosphorus absorption efficiency and serum phosphorus. However, further investigation is required to confirm this *in vivo*.

$1,25OH_2D_3$, also affects phosphorus absorption and is one of three known hormones critically involved in phosphorus homeostasis. $1,25OH_2D_3$ is hydroxylated from the inactive ($25,OHD_3$) to the active form ($1,25OH_2D_3$) in the proximal tubule of the nephron.[56] Upon activation,$1,25OH_2D_3$ has systemic effects, including in the intestinal cells where it binds to the vitamin D receptor (VDR), a ligand-dependent nuclear transcription factor, leading to enhanced NaPi-2b expression and greater phosphorus absorption efficiency. This is

observed across animal studies. In Holtzman rats, administration of exogenous $1,25OH_2D_3$ led to greater jejunal phosphorus flux compared with rats depleted of $1,25OH_2D_3$.[47] Further, in a recent study, mice treated with $1,25OH_2D_3$ had 10 times higher jejunal phosphorus flux under low phosphorus conditions, which was also accompanied by significantly higher NaPi-2b protein expression compared with vehicle treated mice.[48] While $1,25OH_2D_3$ plays an important role in increasing phosphorus absorption, active transport of phosphorus can also be upregulated by a low phosphorus diet independently of the actions of $1,25OH_2D_3$.[57,58] This was discovered through the use of VDR KO rodent models. Segawa et al.[58] found that both VDR $^{-/-}$ and VDR $^{+/+}$ mice had increased phosphorus uptake and Napi-2b protein expression in response to a low phosphorus diet and found no significant response differences between the two groups.

Much of what is known about transcellular phosphorus absorption is based on studies using *in vitro* rather than *in vivo* absorption techniques. Recent studies demonstrate distinct differences in absorption between *in vitro* and *in vivo* methods. Marks et al.[30] conducted absorption tests from the jejunum of healthy male Sprague Dawley rats using everted sac (*in vitro*) and *in situ* ligated loop (*in vivo*) techniques and found that under low luminal phosphorus concentrations (0.1 mM), sodium-dependent phosphate transport accounted for 73% of total phosphorus uptake *in vitro* and only 32% *in vivo*. Similar results were observed by Vorland et al.[59] in which 33% of total phosphorus absorbed following an *in situ*

ligated loop absorption experiment was sodium-dependent. Taken together, these data indicate that *in vitro* absorption methods may not accurately represent transcellular absorption *in vivo*. Additionally, these data suggest that paracellular transport contributes to the majority of phosphorus absorption regardless of luminal phosphorus concentration.

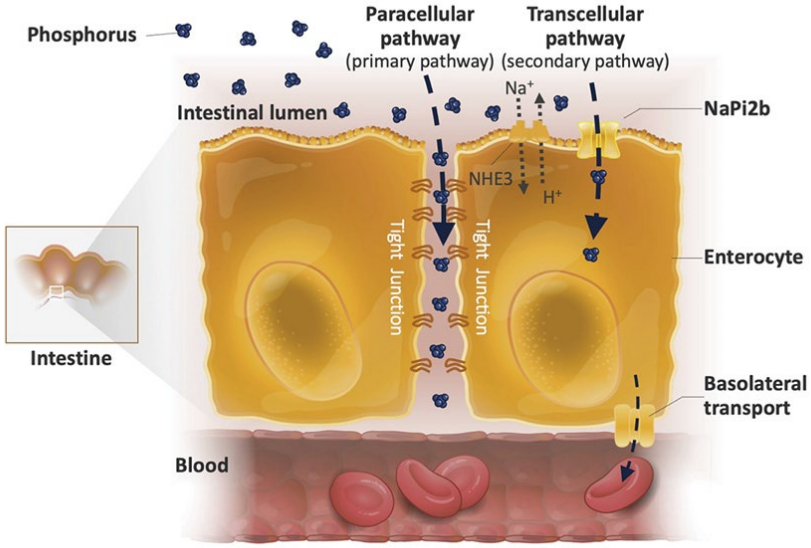

Figure 1.2. Intestinal phosphorus absorption via paracellular and transcellular pathways

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Interestingly, intestinal phosphorus absorption appears to be similar between individuals with normal and impaired kidney function. As those with impaired kidney function have a reduced capacity to excrete serum phosphorus, one would hypothesize that phosphorus homeostasis would be maintained through compensatory mechanisms at the intestine. However, available evidence

does not support this hypothesis. In a study[61] using *in situ* ligated loop absorption methods, there was no difference in jejunal phosphorus uptake in 5/6 nephrectomized compared with sham rats. Using the same *in situ* ligated loop absorption technique, Vorland et al.[59] observed only a slight increase in intestinal phosphorus absorption efficiency in the Cy/+ rat model of progressive CKD-MBD compared to normal littermates. In addition, no difference in phosphorus absorption was observed in CKD compared with healthy rats after an acute oral phosphate challenge.[62] Similar results have been observed in humans.[63,64] Despite lower $1,25OH_2D_3$ in patients with CKD, Stremke et al. observed no difference in fractional phosphorus absorption between patients with stage 3-4 CKD and healthy adults.[63] This underscores the need for further investigation of intestinal phosphorus absorption in CKD.

Renal Phosphorus Excretion and Reabsorption

The kidneys are the major regulators of phosphate homeostasis.[33,65] Phosphorus is freely filtered by the glomerulus and under normal conditions is 75-90% reabsorbed.[33,65,66] Excess serum phosphorus is excreted and 100% recovered in the urine in humans with normal kidney function.[67] Phosphorus reabsorption occurs primarily in the proximal tubule of the nephron via transcellular absorption by sodium dependent phosphate transporters NaPi-2a (SLC34A1), NaPi-2c (SLC34A3), and PiT-2 (SLC20A2) (**Figure 1.3**).[33,68,69] NaPi-2a accounts for ~70% of phosphate reabsorption in rodent models and has similar mRNA expression patterns across species (mice, rats, and humans).[70,71]

However, in humans, both NaPi-2a and NaPi-2c have been shown to play a critical role in renal phosphorus reabsorption.[72]

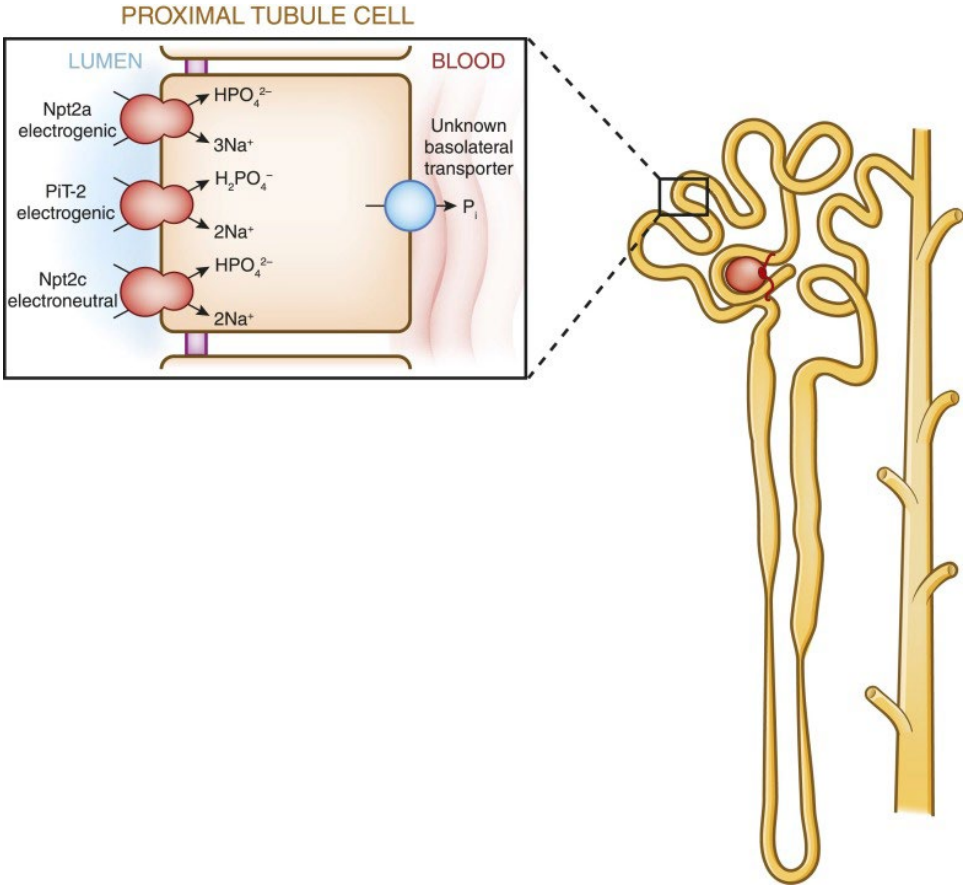

Figure 1.3. Renal phosphate handling in the proximal tubule of the nephron

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Dietary phosphorus load is a major factor affecting renal phosphate handling. Evidence from rodent models show that a low phosphorus diet leads to increased renal phosphate transporter expression and phosphorus reabsorption whereas a high phosphorus diet leads to decreased renal phosphate transporter expression and increased urinary phosphorus excretion.[57,73–77] In healthy

Sprague-Dawley rats, sodium dependent phosphate transport activity was >3-fold higher in renal BBMV of rats on a low phosphorus (0.1%) compared with high phosphorus (1.2%) diet.[76] Further, Villa-Bellosta et al.[73] observed an increase in expression of NaPi-2a, NaPi-2c, and PiT2 in response to a low phosphorus (0.1%) compared with a normal (0.6%) and high (1.2%) phosphorus diet. Other factors that affect renal phosphate handling include FGF23 and PTH and are described below.

Hormonal Regulation of Phosphorus

Hormonal regulation of phosphorus is mediated by three major hormones, FGF23, PTH, and $1,25OH_2D_3$. FGF23 and PTH are both considered phosphaturic hormones as they primarily effect renal phosphate handling, causing an increase in urinary phosphorus excretion and decrease in serum phosphorus.[78–82] The main role of $1,25OH_2D_3$, in phosphorus homeostasis is to increase absorption, leading to increased serum phosphorus. As $1,25OH_2D_3$, was described in the previous section, this section will focus on FGF-23 and PTH.

FGF23 was the most recently discovered phosphaturic hormone, with its effects on phosphorus being characterized in the early 2000s.[83,84] KO mouse models of FGF-23 exhibited abnormal bone development while mice with FGF-23 overexpression exhibited severe phosphate wasting.[78,79,85–87] When kidney function is normal, excess phosphorus is excreted into the urine through the actions of FGF23. Though phosphate sensing is not yet fully understood, conditions of high phosphorus loads cause release of FGF23 produced in

osteoblasts and osteocytes.[88] Subsequently, FGF23 acts on the kidney through its co-receptors, FGF23 receptors (isoforms 1c, 3c, and 4) and alpha-Klotho, in turn, causing internalization and degradation of the renal phosphate transporters NaPi-2a and Napi-2c.[79,83,89,90]

PTH also plays a critical role in maintaining phosphorus homeostasis. Similar to the actions of FGF23, PTH influences renal phosphate transporter localization and expression.[82,91–95] When serum phosphorus is elevated, PTH is secreted into circulation via the parathyroid gland. Following release, PTH binds to the parathyroid hormone 1 receptor (PTHR1), leading to internalization and degradation of the sodium phosphate transporters.[94,96,97] Similar to FGF23, animal models helped determine the mechanism by which PTH impacts renal phosphate handling.[82,95] Notably, alterations in NaPi-2a in response to PTH have been observed to occur as soon as 15 minutes after PTH administration.[81]

PTH is also well known for its role in maintaining calcium homeostasis. Briefly, when serum calcium is low, less calcium is available to bind the calcium sensing receptor (CaSR), found on the parathyroid gland. This causes PTH to be released into circulation and then acts on the bone and kidney to activate mechanisms to increase serum calcium (i.e., bone resorption and increasing renal reabsorption efficiency).[98–100] Once serum calcium returns to the normal range, PTH is reduced due to inhibitory feedback mechanisms.

Importantly, the mechanism by which high serum phosphorus is sensed to release FGF23 and PTH is largely unknown and continues to be investigated. Phosphate sensing for PTH regulation was recently observed to occur via the CaSR on the parathyroid gland.[101] In a preclinical study, Centeno et al.[101] incubated parathyroid glands from CaSR KO and control mice with varying concentrations of phosphate and found that 2 mM and 3 mM phosphate concentrations (representing hyperphosphatemia in humans), led to a significant increase in PTH secretion in control mice whereas no response was observed in CaSR KO mice. This suggests that at least under high phosphate conditions, the CaSR may be a vital component in sensing phosphate. Contrarily, the way in which excess phosphorus is sensed for FGF23 regulation remains largely unknown. One hypothesis by Takashi et al.,[102] is that phosphate may be sensed by the FGF receptor 1 (FGFR1). This hypothesis was developed as their group has shown that excess phosphate initiates a signaling transduction pathway through FGFR.[103] This leads to the activation of a *galnt3* gene product that affects the regulation of active FGF23.[103] However, further study on phosphate sensing for both PTH and FGF-23 regulatory action is required.

Similar results observed in rodent studies of the effect of dietary phosphorus load on the regulation of PTH and FGF-23 have also been demonstrated in humans.[62,67,104,105] In a crossover study[104] of 8 healthy adults, it was observed that serum PTH was significantly higher as early as 1 hour following consumption of a 800mg and 1200mg phosphorus test meal compared

with a 400 mg test meal. Interestingly, serum FGF23 was only elevated 8 hours after consumption of the 1200mg phosphorus test meal. These results were corroborated by Turner et al.[62] in which a 500 mg phosphorus oral challenge lead to a significant increase in PTH at 30 minutes post consumption, whereas no change in FGF23 was observed over the 180 minute period. Taken together, these data suggest that PTH is an acute responder to dietary phosphorus load while FGF-23 is a chronic responder.

It is important to note that these hormones do not work in isolation from one another (**Figure 1.1**). As an acute responder to dietary phosphorus load, PTH is secreted first by the parathyroid gland and stimulates renal phosphorus excretion. Further, PTH stimulates $1,25OH_2D_3$ production at the kidney and FGF-23 production at the bone.[23] $1,25OH_2D_3$ further stimulates FGF-23 production and can act to increase phosphorus absorption through regulation of intestinal phosphate transporter expression. Importantly, $1,25OH_2D_3$ is self-inhibiting through its suppression of 1α-hydroxylase (CYP27B1) and has inhibitory effects on PTH production.[106] In addition, FGF-23 exerts effects to suppress both PTH and $1,25OH_2D_3$ while also acting to increase renal phosphorus excretion.[23] These complex regulatory mechanisms of FGF-23, PTH, and $1,25OH_2D_3$ maintain phosphorus homeostasis in health. However, abnormalities in phosphorus metabolism and dysregulation of these hormones are common in CKD.

Characteristics of CKD-Mineral and Bone Disorder

Declining kidney function is associated with numerous health consequences including abnormal phosphorus handling (**Figure 1.4**). But, the compensatory actions of elevated FGF-23 and PTH and decreased 1,25OH_2D_3 maintain serum phosphorus within the normal range until late-stage CKD where these mechanisms are no longer sufficient and hyperphosphatemia occurs (**Figure 1.5**).[107]

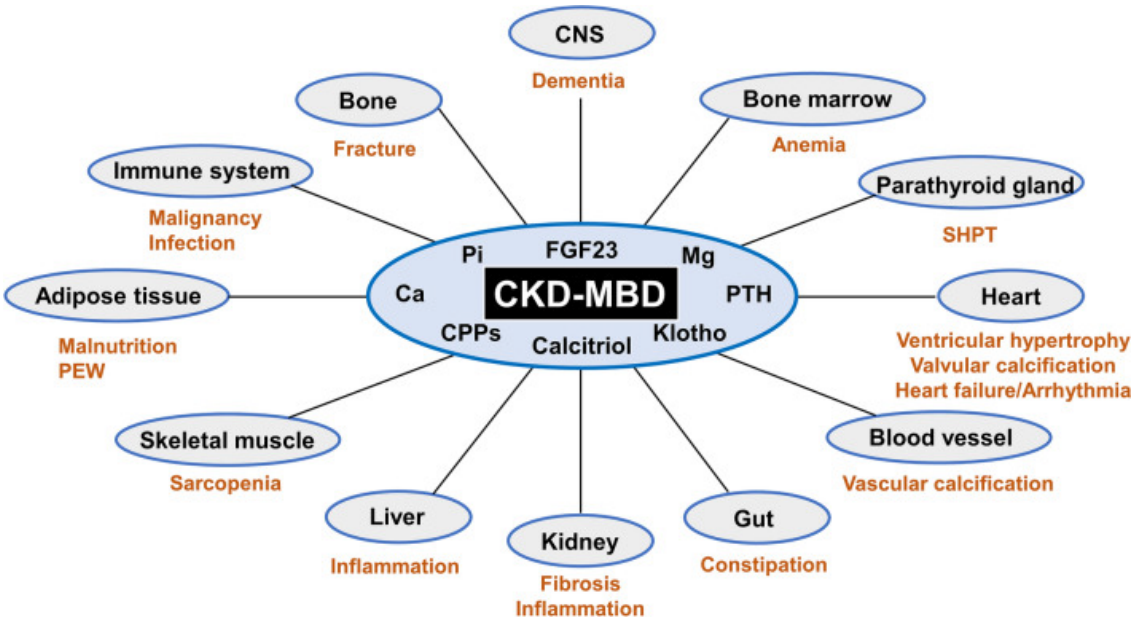

Figure 1.4. CKD-MBD and its associated complications

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These abnormalities in phosphorus metabolism are central to the development of CKD-mineral and bone disorder. CKD-MBD is defined by the Kidney Disease Improving Global Outcomes (KDIGO)[109] as: "*A systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a*

combination of the following: abnormalities of calcium, phosphorus, PTH or vitamin D metabolism, abnormalities in bone turnover, mineralization, volume, linear growth, or strength, and/or vascular or other soft tissue calcification". This is detrimental to the health and quality of life in adults with CKD as it increases their risk of bone fragility fractures, vascular and soft tissue calcification and death (**Figure 1.4**).[15,110–112]

Elevations in FGF-23 and PTH are major factors that affect these clinical outcomes. FGF-23 is the first hormone to respond to disturbances in phosphorus metabolism, occurring as early as stage 2 CKD and remains persistently elevated as kidney function continues to decline (**Figure 1.5**).[107,113] This exacerbates CKD-MBD complications including coronary artery and abdominal aortic calcification, and left ventricular hypertrophy.[114–118] In fact, one group[115] found that a serum FGF-23 level of 277 pg/mL could be used as a diagnostic value for abdominal aortic calcification with 90.7% accuracy in a group of patients on hemodialysis. Another common complication observed in CKD-MBD is secondary hyperparathyroidism (SHPT), characterized by elevated PTH, and another contributing complication to worsened cardiovascular and bone outcomes.[119] In a recent prospective study, Xu et al.[120] found that the development of SHPT was associated with a 2.2-fold higher risk of major adverse cardiovascular events, 1.8-fold higher relative risk of fracture, 5-fold higher risk of CKD progression, and 1.4-fold higher risk of death. Thus, efforts must be made to prevent or mitigate these detrimental health complications.

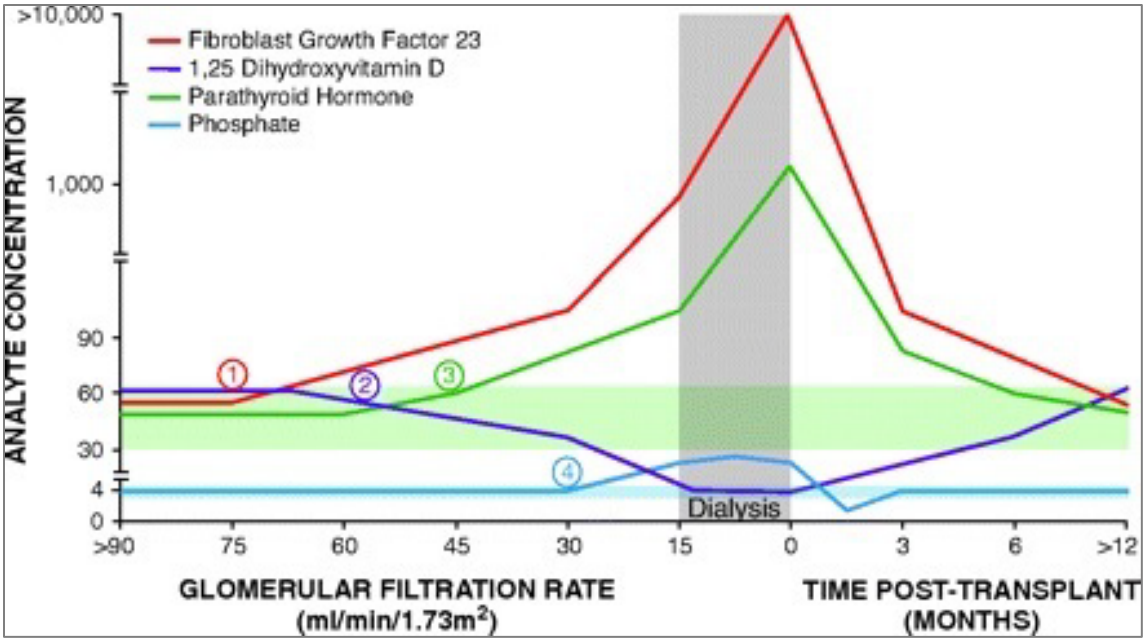

Figure 1.5. Abnormalities in phosphorus metabolism and hormonal regulation with progressive kidney function decline

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Dietary Recommendations for the Management of CKD

To aid in the management of CKD and prevent the complications associated with CKD-MBD, patients are advised to follow specific dietary recommendations, which vary depending on the stage of CKD. Of particular emphasis is the amount of dietary protein and phosphorus patients with CKD should consume.

Dietary Protein

The most recent (2020) update to the Kidney Disease Outcomes Quality Initiative (KDOQI) Clinical Practice Guideline for Nutrition in CKD[121] recommends a protein intake of 0.55-0.6 g/kg/d for stage 3-5 CKD (non-dialysis without diabetes), which is considered a low protein diet. This is aimed at preserving

kidney function as consumption of a high protein diet may lead to glomerular hyperfiltration and increased azotemia.[122–124] In addition, following a low protein diet can aid in phosphorus control as protein-rich foods are also high in phosphorus. Once a patient with CKD starts hemo- or peritoneal dialysis, their protein needs change due to greater dialysis related protein loss and protein catabolism that puts these patients at a higher risk for protein energy malnutrition.[125–127] To prevent this, protein recommendations increase to 1.0-1.2g/kg/d.[121,127]

Notably, the recommendation for the type of protein patients with CKD should consume was recently updated. The new guidelines state that there is insufficient evidence to recommend a particular protein type (plant vs animal) for patients with CKD.[121] This is contrary to the previous 2000 KDOQI nutrition guidelines[128] in which it was recommended that at least half of protein consumed be of 'high biological value'. Biological value is defined as the efficiency by which the body is able to utilize protein for growth and maintenance.[129,130] Foods that contain all nine essential amino acids are considered of high biological value. This is generally found in animal protein (e.g., beef, milk, eggs) rather than plant-based protein (e.g., nuts, pulses, beans).[130] However, when certain plant-based proteins or ingredients are consumed in combination (the concept of complementary proteins) they too, can yield a high biological value by providing all 9 essential amino acids.[131,132]

Dietary Phosphorus

As phosphorus is central to CKD-MBD, it is often recommended that patients with CKD follow a phosphorus restricted diet. In the previous (2003) KDOQI Clinical Practice Guidelines for Bone Metabolism and Disease in CKD,[133] it was recommended that dietary phosphorus intake be restricted to 800-1000mg/d. However, in the most recent KDOQI Nutrition in CKD guidelines, it is recommended that dietary phosphorus intake be adjusted to maintain serum phosphorus within the normal range for patients with stage 3-5D CKD.[121] In addition to these updates, both the 2020 KDOQI Nutrition in CKD guidelines and 2017 KDIGO CKD-MBD guidelines propose taking phosphate source into consideration when making dietary recommendations for patients with CKD.[8,121] This is based on evidence that phosphorus absorption differs depending on phosphate source. Absorption of phosphorus is thought to be lowest from plant foods, higher from animal foods, and highest from inorganic phosphate additives.[22,134–136] Further explanation of this will be provided in the sections to follow.

In combination with a restricted phosphorus diet, phosphate binder medications are often prescribed. Phosphate binders were first introduced in the 1970s and have spanned a variety of categories including aluminum-based, calcium-based and most recently, non-calcium based.[137–139] They can effectively reduce serum phosphorus by binding and limiting the amount of phosphorus that is absorbed and increasing the amount excreted in the feces.[139] Phosphate

binders have been shown to have an average binding capacity of ~250 mg phosphorus per day for typical doses which can be increased to ~450 mg when a combination of two binders are used.[140–143]

Many studies provide evidence of beneficial effects of phosphorus restriction and binder use on reducing serum phosphorus, PTH, and FGF23.[144–148] However, there is limited evidence to suggest that following a low phosphorus diet prevents adverse clinical endpoints such as CVD, fracture risk and death.[149] Moreover, availability of these endpoints are generally lacking in the literature. A recent multicenter, double-blind randomized controlled trial (RCT) of 278 patients with stage 3b-4 CKD, found that phosphorus reduction through the use of lanthanum carbonate over 96 weeks did not affect vascular calcification.[150] Further, in a systematic review of dietary interventions aimed at improving mineral and bone disorder outcomes in patients with CKD, Liu et al. found that of 9 included studies, no studies reported on cardiovascular or fracture events and one study reported on death.[151] As following a low phosphorus diet can be strenuous and burdensome to patients with CKD, it is of utmost importance that further investigation of the effects of phosphorus restriction on clinical endpoints be conducted.

Challenges Associated with Current Management Strategies for the Control of Phosphorus in CKD

There are numerous challenges that patients with CKD face when following a phosphorus restricted diet or taking phosphate binders. Phosphorus

is widespread in the food supply, making it difficult to reduce phosphorus intake. Data from the 2017-2018 National Health and Nutrition Examination Survey – What We Eat in America (NHANES WWEIA) shows that the average phosphorus intake for adults ≥ 20 years of age is 1600 mg/day for men and 1199 mg/day for women, averaging nearly double the recommended dietary allowances (RDA) (set at 700 mg/d for adults).[152,153] However, this is still below the current DRI tolerable upper intake level (UL) of 4,000 mg/d.[153] Notably, the DRI for phosphorus has not been updated since 1997 and was established based on serum phosphate as a biomarker of nutritional adequacy, which may not be a reliable indicator as serum phosphate is relatively well-controlled through hormonal regulation.[154] When the DRI for the UL was set, most available evidence on the harms of consuming excess phosphorus were from studies performed on animal models.[153] Since that time, FGF-23 was discovered as a major hormone involved in phosphate homeostasis and studies have shown an increased risk of cardiovascular endpoints even with serum phosphorus in the normal range in both the general population and those with CKD.[155–158]

Most food sources contain some amount of phosphorus, but protein foods and foods with phosphate-containing additives (deli meat, cola, canned fish, baked goods etc.) have the highest amounts.[159] Notably, ~76% of total dietary phosphorus consumption comes from grains, meat and milk.[160] However, products with phosphate-containing food additives also considerably impact dietary phosphorus burden.

Phosphate-containing food additives are commonly used as flavor enhancers, leavening and hydration agents, and emulsifiers.[161] Their prevalence in the food supply has been widely studied[162–164] with one group finding that nearly half (44%) of top-selling grocery items contained a phosphate additive and contributed an average ~67 mg more total phosphorus per serving than those without phosphate additives.[162] Similar results have been observed for meat, poultry and fish products also containing phosphate additives.[163] This greatly impacts daily phosphorus consumption with studies estimating ~600-736 mg difference in phosphorus per day when consuming meals with phosphate-containing additives versus meals without.[162,164] The high phosphorus burden from these additives may negatively affect serum phosphorus, especially in patients with CKD. Indeed, in a randomized clinical trial of hemodialysis patients, those who consumed a diet without phosphate additives had a significant reduction in serum phosphorus compared with controls and ~70% reached target serum phosphorus levels of ≤5.5 mg/dL.[165] However, it remains to be determined if this reduction in serum phosphorus improves clinical outcomes (CVD, fracture risk, etc.).

Phosphorus is also found as an excipient in some medications, but this is often overlooked as a source contributing to overall phosphorus burden.[166] Unfortunately, the contribution of phosphorus from medications is challenging to quantify and varies considerably from person to person. In one study, phosphorus was found to range from ~0-112 mg per dose of medications

commonly prescribed.[167] While, in another study, evaluating the five most commonly prescribed drug classes to dialysis patients, it was found that if all five medications were prescribed this could increase a patient's daily phosphorus burden by ~400 mg.[168]

An additional challenge is that phosphorus is not required to be reported on the Nutrition Facts Label. Not only does this make it difficult for healthcare practitioners to make appropriate dietary recommendations for patients with CKD, but it also makes it very difficult for patients to accurately follow a reduced phosphorus diet. In 2016, the Food and Drug Administration (FDA) updated the Nutrition Facts Label, which had not been revised for over 20 years.[169] However, adding phosphorus to the Label was forgone.[170] This leaves healthcare practitioners and patients with CKD to use nutrient databases to estimate phosphorus content in foods. These estimates may not reflect actual phosphorus intake as nutrient databases are often incomplete and inaccurate.[171–175] Of popular beverages, it was observed that 39% did not have an exact match in the Nutrient Data Systems for Research (NDSR) database and of the products that were matched, 78% underestimated phosphorus compared with chemically analyzed values.[173,174] This is similar to observations by others in which phosphorus content were reported to be underestimated in 15-25% of various food products[171] and ~40% in a mixed meal.[172] Interestingly, although manufacturer reporting of phosphorus is voluntary, phosphorus data were found

to be available for 30% of products and were most similar to chemically analyzed values.[174]

Challenges and health consequences associated with phosphate binder adherence should also be considered when utilized as a strategy for phosphorus management. Patients with CKD already experience a high pill burden, which is only exacerbated by the use of phosphate binders.[176–179] In one study,[140] it was found that on average, 8 phosphate binder tablets were taken per day (range 1-27 tablets/day). In addition, the composition of phosphate binders may affect health outcomes. Specifically, calcium-based phosphate binders have been shown to increase the risk of cardiovascular events and vascular calcification in patients with CKD.[180,181]

Overall, it is evident that there are many obstacles that healthcare practitioners and patients with CKD are faced with when recommending and following a reduced phosphorus diet or taking phosphate binders. Overcoming these challenges is burdensome and often leads to limited adherence to these recommendations.[182,183] Therefore, new approaches must be explored for the control of phosphorus in patients with CKD.

A New Approach for the Management of Phosphorus Control in CKD

One approach of growing interest for the management of dietary phosphorus and other health consequences in CKD is greater consumption of plant-based foods. Plant-based diets offer a diverse nutritional profile as they are rich in vitamins, minerals, fiber, and mono- and poly-unsaturated fatty acids and

can have a variety of health benefits (**Figure 1.6**). Numerous studies are available that demonstrate the benefit of plant-based diets on inflammation, insulin resistance and sensitivity, and cardiovascular disease (**Figure 1.6**).[184–189] For example, in a systematic review and meta-analysis of RCTs, Li et al.[184] found that substitution of animal protein for ~30g/d plant-based protein reduced low-density lipoprotein (LDL) and non-high-density lipoprotein (non-HDL) cholesterol. Moreover, greater adherence to a plant-based protein diet was found to lower the risk of CVD in a systematic review and meta-analysis of 7 prospective cohort studies.[185]

Plant-based diets are also associated with reduced kidney disease incidence, suggesting this dietary pattern has a protective effect on kidney function. In a prospective study of 1,639 adults, a 70% decrease in incidence of CKD was observed in the highest compared with the lowest tertile of plant-based protein consumption.[190] Similarly, Asghari et al.[191] found that the highest compared with the lowest tertile of consumption of a lacto-vegetarian eating pattern was associated with a 43% reduced odds of CKD incidence. Specifically, higher intake of nuts, legumes and low fat dairy were observed to be associated with lower CKD risk.[192] In addition, plant-based diets have been proposed to improve metabolic acidosis, alter the gut microbiome, reduce uremic retention solutes, and lower the risk of mortality in patients with CKD.[193–201] Therefore, recommending greater consumption of a plant-based diet may be a viable option to mitigate the health complications observed with CKD-MBD.

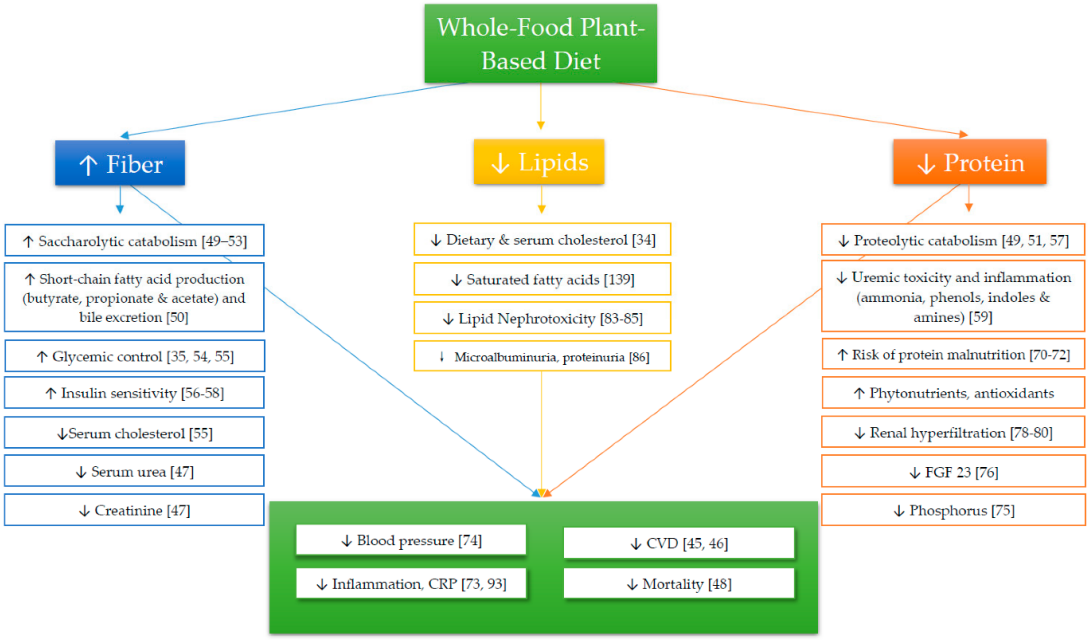

Figure 1.6. Potential benefits and harms of consuming a plant-based diet for patients with CKD

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Importantly, plant-based foods are thought to have lower bioavailable phosphorus and could be utilized as an approach to control phosphorus intake in CKD. Current understanding is that phosphorus bioavailability is lowest in plant sources (~10-50%), higher in animal sources (40-60%), and highest in inorganic phosphate additives (80-100%).[22,134–136] Phosphorus bioavailability of a mixed meal is thought to be ~55-70% bioavailable.[18] However, limited evidence is available investigating *in vivo* phosphorus bioavailability of food and food products for humans. In fact, most available studies simulate human digestion using *in vitro* digestion experiments, which determine phosphorus bioaccessibility rather than bioavailability. While bioavailability and bioaccessibility are often used

interchangeably, it is important to recognize the difference between the two terms. Bioavailability refers to the amount of a nutrient that is digested, absorbed, and available for use by the body whereas bioaccessibility refers to the amount of a nutrient that is digested and accessible for absorption.[202,203] Interestingly, numerous studies are available on phosphorus bioavailability of poultry feed. Research has been done in this area to understand phosphorus requirements and enhance phosphorus utilization in poultry in an effort to reduce the environmental ramifications of excess phosphorus excretion in poultry fecal matter.[204–207]

As mentioned above, phosphorus bioaccessibility varies considerably depending on phosphorus source. In plant-based foods, a majority of phosphorus is found in phytic acid, contributing to the lower phosphorus bioaccessibility of these foods. Phytic acid, also known as myo-inositol-1,2,3,4,5,6-hexakis phosphate[208,209], is the main storage form of phosphorus in plant-foods, accounting for ~50-82% of total phosphorus[210,211]. It is also labeled an "antinutrient" as it complexes with numerous minerals (e.g., calcium, iron, magnesium, zinc etc.), preventing their absorption.[212–214] Phytase is required to efficiently hydrolyze phytic acid, in turn, releasing phosphorus for absorption. However, humans have minimal amounts of phytase in the gastrointestinal tract and do not intrinsically produce phytase.[215] Thus, phosphorus remains bound in phytic acid and is unable to be absorbed. This is contrary to inorganic

phosphorus, commonly used in phosphate additives, where bioaccessibility is only dependent on the solubility of the inorganic salt.

Despite the relatively sparse literature on phosphorus bioaccessibility, two studies published by Karp et al.[216,217] provide data for bioaccessible phosphorus on a number of plant foods, beverages, dairy, and meat products using an *in vitro* method for human digestion. They found that phosphorus bioaccessibility of plant foods ranged from ~6-42%. Phosphorus bioaccessibility of dairy products including milk and a variety of cheese products ranged from ~49-111% and phosphorus bioaccessibility of meat products including uncooked sausage, pork, chicken, and trout ranged from ~70-107%. Furthermore, beverages that contained phosphate-additives (n=5) had nearly 100% bioaccessible phosphorus (84-100%). The same research group[218] determined phosphorus bioaccessibility of rye, wheat, and barely cereal products. They found the lowest bioaccessible phosphorus in barley grit (29%) and the highest in whole grain rye sourdough (99%).

While these data align with proposed values for phosphorus bioaccessibility,[22] it remains unclear how this may differ *in vivo.* In a study of healthy adults, Scanni et al.[67] characterized the acute response to either an intravenous (I.V.) or nasoduodenal phosphate load. As expected, 100% of phosphorus from the I.V. infusion was recovered in the urine whereas only 73% of the nasoduodenal administered load was recovered, suggesting that inorganic phosphate additives may not be 100% bioavailable.[67] Further, in a commentary

review comparing the proportion of dietary phosphorus excreted in the urine from clinical trial data, St. Jules et al.[136] found that urinary phosphorus excretion from diets with phosphate additives was similar to or lower than diets without phosphate additives. Notably, these studies use 24-hour urinary phosphorus as a proxy for phosphorus absorption, but this may not accurately reflect true phosphorus absorption if an individual is not in phosphorus balance.[136,219] This is especially true for patients with CKD as Stremke et al.[219] show that 24-hour urine phosphorus does not relate to dietary phosphorus intake or absorption in 8 patients with stage 3-4 CKD following a tightly controlled two-week balance study. In a more recent study, Stremke et al.[63] show that 24 hour urinary phosphorus excretion was not correlated with fractional intestinal phosphorus absorption or the absolute amount of phosphorus absorbed in both the CKD and healthy adult group. This emphasizes the need to perform studies aimed at determining *in vivo* phosphorus bioavailability of different phosphate sources to better inform clinical guidelines for dietary recommendations in CKD.

While there are many proposed benefits for the consumption of a plant-based diet for patients with CKD, the potential harms of such a diet must also be considered. One such harm is the risk of hyperkalemia as plant foods are generally high in potassium. However, it is noted in the 2020 KDOQI Clinical Practice Guidelines for Nutrition[121] that there is limited clinical trial evidence available showing the effect of dietary potassium consumption on clinical outcomes in patients with CKD. Additionally, in a recent prospective cohort study,

D'Alessandro et al.[220] found that the prevalence of hyperkalemia was no different between CKD patients on a normal protein, low protein, or plant-based vegetarian low protein diet. Another potential harm is the risk of protein energy wasting or protein energy malnutrition as patients with CKD on dialysis require higher amounts of protein which may not be easily achievable through consumption of plant-based protein. In a critical review of the impact of protein source on nutrition status in adults with CKD, Picard et al.[221] conclude that available evidence does not show that higher plant protein consumption leads to worse nutritional outcomes. Other potential harms or risks of consuming plant-based protein or plant-based protein diets must be further elucidated.

Conclusion

Dietary recommendations for patients with CKD are quite complex and can be strenuous and burdensome to follow. Understanding how bouts of non-adherence to these dietary recommendations affect health outcomes is paramount to creating appropriate guidelines. Moreover, gaining a better understanding of how other dietary approaches are tolerated and how they impact health outcomes for patients with CKD is critical to continue to advance the field forward. Therefore, the aim of this dissertation is to evaluate how dietary phosphorus amount, source, and bioaccessibility affect intestinal phosphorus absorption and health outcomes in CKD.

Chapter 2: Acute High Dietary Phosphorus Following Low Phosphorus Diet Acclimation Does Not Enhance Intestinal Fractional Phosphorus Absorption in Nephrectomized Male Rats

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Abstract

Dietary phosphorus restriction and phosphorus binders are commonly prescribed for patients with chronic kidney disease (CKD). However, occurrences of non-adherence to these interventions are common. As low phosphorus (LP) diets have been consistently experimentally shown *in vitro* to increase intestinal phosphorus absorption efficiency, a bout of non-adherence to diet or binders may cause an unintended consequence of enhanced intestinal phosphorus absorption. Thus, we aimed to determine the effect of a single bout of high phosphorus (HP) intake following acclimation to a LP diet. Male Sprague-Dawley rats with 5/6 nephrectomy (n=36) or sham operation (n=36) were block-randomized to 1 of 3 diets: LP (0.1% P w/w), HP (1.2%), or LP followed by acute HP (LPHP 0.1% then 1.2%). Phosphorus absorption tests were conducted using ^{33}P radioisotope administrated by oral gavage or I.V. Although the overall two-

way ANCOVA model for intestinal fractional phosphorus absorption was non-significant, exploratory comparisons showed intestinal fractional phosphorus absorption efficiency tended to be higher in rats in the LP compared to HP or LPHP groups. Rats in the HP or LPHP groups had higher plasma phosphorus compared with rats in the LP group, but the LPHP group was not different than the HP group. Gene expression of the major intestinal phosphate transporter, NaPi-2b was lower in the jejunum of rats in the LPHP group compared to rats in the HP group but not different in the duodenum. These results demonstrate that an acute HP load following acclimation to a LP diet does not lead to enhanced intestinal fractional phosphorus absorption efficiency in 5/6 nephrectomized male rats. These data provide evidence against the notion that dietary phosphorus restriction or binder use adversely increases absorption efficiency following a single instance of dietary or binder non-adherence. However, other adverse consequences of fluctuating dietary phosphorus intake cannot be ruled out.

Introduction

Individuals with chronic kidney disease (CKD) develop disturbances in mineral metabolism as the disease progresses.[107,222–224] These disturbances lead to CKD-mineral bone disorder (CKD-MBD) including increased vascular calcification,[225,226] bone fragility fractures,[227,228] and mortality.[15,229] Abnormal phosphorus metabolism drives the development of CKD-MBD. In the absence of pathology, phosphorus homeostasis is maintained through the actions of the intestine, bone, parathyroid gland, and kidney.[18] Healthy kidneys are able to fully compensate for increased phosphorus loads by increasing urinary phosphorus excretion.[67] However, as kidney function declines, urinary phosphorus excretion becomes compromised, contributing to abnormal phosphorus handling leading to elevated plasma fibroblast growth factor-23 (FGF-23), elevated parathyroid hormone (iPTH), lower 1,25-dihydroxyvitamin D (1,25D), and eventual elevated plasma phosphorus.[107] Intestinal absorption of dietary phosphorus is a major component of overall phosphorus balance. Therefore, common approaches used to manage hyperparathyroidism and hyperphosphatemia include dietary phosphorus restriction and the use of phosphate binder medications.[18] However, adhering to a low-phosphorus diet is challenging as phosphorus is widespread in the food supply, including naturally occurring sources in protein and grain foods, and in food products with phosphate-containing additives.[160] Further, measurement and disclosure of phosphorus content in foods is not required on the Nutrition Facts Label, making it difficult for patients with CKD to make

informed food selections and for healthcare practitioners to make accurate recommendations.[170] Thus, bouts of non-adherence to low phosphorus diets are common. In fact, in an integrative review aimed at determining dietary adherence in late-stage CKD, Lambert et al.[182] reported that adherence to a low phosphorus diet ranged from 43.5-84.5% across 15 studies.

There is sparse literature investigating the physiological effects of bouts of dietary phosphorus restriction non-adherence on intestinal phosphorus absorption. However, low phosphorus diets have been shown to increase expression of the intestinal sodium-phosphate co-transporter (NaPi-2b), which is associated with *in vitro* measures of greater brush border membrane vesicle phosphorus uptake[34,39,43,230,231] or increased phosphate flux.[232] This suggests that dietary phosphorus restriction or binder use may have an adverse consequence by enhancing intestinal phosphorus absorption if there is one or multiple bouts of non-adherence with diet or binders. A study by Giral et al.[39] measured *in vitro* intestinal phosphorus uptake efficiency in isolated brush border membrane vesicles (BBMV) from healthy rats fed either a low phosphorus diet for the duration of the study, or acutely switched to a high phosphorus diet on the last day. Unexpectedly, the rats acutely switched to the high phosphorus diet had even greater BBMV phosphorus uptake efficiency than the rats that continued the low phosphorus diet in a seemingly maladaptive response. Further, serum phosphorus was 3-fold higher following the acute high phosphorus load compared with rats kept on the low phosphorus diet, and 2-fold higher than rats

that had been on the high phosphorus diet for 7 days in a separate experiment. These data suggest that dietary phosphorus restriction may cause an unintended adverse increase in phosphorus absorption efficiency and serum phosphorus after dietary non-adherence. However, this has not been evaluated in rats with CKD, nor with *in vivo* intestinal phosphorus absorption testing methods.

The primary aim of this study was to test the hypothesis that a single bout of high phosphorus intake following acclimation to a low phosphorus diet in 5/6 nephrectomized male rats will increase intestinal phosphorus absorption. Secondary outcomes included plasma biochemistries related to CKD-MBD and gene expression of the intestinal phosphate transporters, NaPi-2b/*slc34a2*, sodium-dependent phosphate co-transporter 1 (PiT-1/*slc20a1*), and sodium-dependent phosphate co-transporter 2 (PiT-2/*slc20a2*). Based on Giral et al.,[39] we hypothesized that intestinal fractional phosphorus absorption (efficiency) would be highest in rats acclimated to the low phosphorus diet then acutely switched to the high phosphorus diet.[59]

Materials and Methods

Study Design

In a 2 x 3 factorial design study, n=72 commercial male Sprague-Dawley rats (Charles River, Indianapolis, IN, USA) with 5/6th nephrectomy (n=36) or sham operation (n=36) were studied. Rats underwent a two-step 5/6th nephrectomy surgery (at Charles River, Indianapolis, IN) at ~7-8 weeks of age and arrived at the study site at Purdue University (West Lafayette, IN)

approximately 1 week after completion of the second surgery. We received rats in four shipment cohorts of n=18/shipment which included n=9 nephrectomized rats and n=9 sham-operated rats. Rats were block-randomized to one of three dietary treatments within the four shipment cohort blocks and the two disease status blocks, with equal distribution of treatments in each shipment cohort and disease status block. This resulted in n=12/group to one of three dietary treatment groups by disease status (nephrectomy or sham). Group allocation was concealed from investigators handling the rats only during the 3-week acclimatization period, and the study was unblinded once the study diets and experimentation period began. Rats were housed on a 12h light/dark cycle in a temperature and humidity-controlled room. Upon arrival to the study site, rats were group housed (2 rats/cage) in standard solid bottom caging with Aspen bedding for ~2 weeks and thereafter transferred to individual wire-bottom metabolic cages one week prior to starting the diet treatment and through the end of the study. Prior to initiating study diets, rats were fed a non-autoclaved grain and soy-based standard rodent diet (Envigo Teklad 2018, Indianapolis, IN) and received filtered water *ad libitum*. After the 3-week acclimatization period (4 weeks post-surgery and ~12 weeks of age), rats were switched to their randomly-assigned diet treatment of either low phosphorus (LP, total P 0.1%, 0.6% Ca w/w, TD.85010 Envigo Teklad, Indianapolis, IN, USA), high phosphorus (HP, total P 1.2%, 0.6% Ca w/w, TD.85349 Envigo Teklad, Indianapolis, IN, USA) or low phosphorus followed by acute high phosphorus on the last (7^{th}) day (LPHP,

0.1% w/w then 1.2% w/ w). All study diets were non-autoclaved egg-white protein based (**Table A.A.S1**). Access to food was restricted to a daily four-hour feeding window (~8am-12pm) for seven days, with water *ad libitum*. LPHP rats were fed the LP diet on days 1-6 and the HP diet on day 7 (**Figure 2.1**). The diet intervention duration, 4-hour feeding window, and the low (0.1% w/w) and high (1.2% w/w) dietary phosphorus levels were chosen based on the study design of Giral et al..[39] Following the four-hour feeding window on day 7, rats underwent an *in vivo* intestinal phosphorus absorption testing procedure (oral gavage test). Blood draws for biochemical analyses were taken at baseline (day 1, prior to start of study diet, jugular vein draw) and after euthanasia (day 7, abdominal aortic draw). Rats were weighed thrice weekly and food was weighed daily while rats were on the study diets to determine food consumption. This protocol was approved by the Purdue University Animal Care and Use Committee (Protocol Number: 1402001030). The study was pre-registered at Animal Study Registry (10.17590/asr.0000207).

Jugular Catheter Placement

Jugular catheters were placed using aseptic technique as a survival surgery 48 hours prior to intestinal phosphorus absorption efficiency testing. Once anesthetized, rats received pain medication of 2 mg/kg body weight Metacam NSAID injectable or 4-5 mg/kg body weight Carprofen. Rats were then placed in dorsal recumbency, the ventral cervical area shaved, sterilized, and a 2 cm incision was made in the skin over the right jugular vein. The fascia and

underlying cervical muscles were separated by blunt dissection to reveal the jugular vein. The vein was isolated, cut and the catheter was inserted. It was then threaded subcutaneously through the skin to the exit site, at the back of the neck between the shoulder blades. Upon determining patency, incision sites were sutured, and rats were taken off anesthetic. Following completion of surgery, rats were monitored for post-operative complications and catheters were flushed with heparinized saline every 12 hours to maintain patency.

Intestinal Phosphorus Absorption Efficiency

Intestinal phosphorus absorption efficiency was determined by *in vivo* oral gavage absorption testing performed prior to euthanasia. Absorption tests were conducted on rats in order of their randomization. Following the four-hour feeding window on Day 7, n=8 rats/treatment group were orogastric gavaged with 3 mL of a transport solution enriched with 10μCi ^{33}P (^{33}P-orthophosphoric acid, American Radiolabeled Chemicals, Inc, St Louis, MO, USA). The transport solution consisted of Na_2HPO_4 and phosphate buffered saline (PBS) standardized to contain 6.4 mg P for rats on the LP diet treatment and 76.8 mg P for rats on the HP and LPHP diet treatment (corresponding to ~1/3 of total daily P intake on each diet). To account for rate of renal clearance in calculating fractional intestinal phosphorus absorption, n=4 rats/treatment group underwent an intravenous (I.V.) administration of 1 mL sterile saline enriched with 5 μCi ^{33}P via jugular catheter. As the I.V. ^{33}P was administered, these n=4 rats/group were also given an oral gavage of unenriched Na_2HPO_4 and PBS transport solution

containing either 6.4 mg P or 76.8 mg P according to their diet treatment group as described above (**Figure 2.1**). Blood (0.25 mL/sampling) was collected by jugular catheters (placed 48 hours prior to phosphorus absorption testing) at 0, 20, 40, 60, 90, and 120 minutes after dosing with ^{33}P.[62] Blood was transferred to lithium heparin tubes, centrifuged at 10,000g for 10 minutes (Micro 18R, VWR, Radnor, PA, USA) for plasma. Liquid scintillation counting of plasma samples from each time point was performed on a Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA, USA). 100 µL of plasma was counted in 15 mL of EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA, USA). Intestinal fractional phosphorus absorption was determined from the ratio of area under the oral and I.V. plasma ^{33}P curves (AUC_{PO}/AUC_{IV}), calculated for the n=8 rats/group given the oral gavage ^{33}P, using the AUC_{IV} average value from the n=4 rats in the same group given the I.V. ^{33}P.

Tissue and Blood Collection

Rats were euthanized via CO_2 asphyxiation immediately following the phosphorus absorption testing. After euthanasia, the abdominal cavity was opened, and a terminal blood draw was collected from the abdominal aorta and placed in lithium heparin tubes for separation of plasma. The small intestine was excised from the pyloric sphincter to the cecum at the ileo-cecal junction. The excised intestine was flushed with sterile 0.9% NaCl to remove contents and was further cut into sections of duodenum (1 cm distal from the pyloric sphincter to ~10 cm) and jejunum (~10-30 cm). The mucosal layers of the duodenum (~10

cm) and jejunum (~10 cm) were scraped. Mucosal scrapings from each intestinal segment were flash frozen in liquid nitrogen and stored at -80°C for later mRNA extraction.

Intestinal Gene Expression

Total RNA from duodenum and jejunum was isolated using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Target-specific PCR primers were obtained from Applied Biosystems (Foster City, CA, USA): NaPi2b (Slc34a2; Rn00584515_m1); PiT1 (Slc20a1; Rn00579811_m1); PiT2 (Slc20a2; Rn00568130_m1); ribosomal protein, large, P0 (RPLP0, Rn03302271_gH). The gene expression was determined by real-time PCR using TaqMan gene expression assay system (TaqMan MGP probes, FAM dye-labeled; Applied Biosystems, Foster City, CA, USA) using ViiA 7 systems. The cycle number at which the amplification plot crosses the threshold was calculated (CT), and the $\Delta\Delta$CT method was used to analyze the relative changes in mRNA expression and normalized by RPLP0 [233].

Plasma Biochemistries

Plasma was stored at -80°C and thawed prior to biochemical analyses. Plasma blood urea nitrogen (BUN), calcium, and phosphorus were determined by colorimetric assays (Point Scientific, Canton, MI, USA), iPTH and iFGF23 by enzyme-linked immunosorbent assay (ELISA) (Quidel Corporation, San Diego, CA,USA), and 1,25D by enzyme immunoassay (EIA) (Immunodiagnostic Systems, The Boldons, UK).

Statistics

Appropriate sample size was calculated based on phosphorus absorption data in 5/6th nephrectomized rats from Marks et al.[61] Based on this, a sample size of n=8 was deemed sufficient to detect a 30% difference between groups for intestinal phosphorus absorption efficiency (β=0.80, α=0.05). Statistical analysis was performed using Statistical Analysis Software (SAS) version 9.4 (SAS Institute, Cary, NC). Two-way ANCOVA was performed for all outcomes with cohort as a covariate (4 cohorts), main effects for health status (2 levels: CKD and sham), diet treatment (3 levels: LP, HP, LPHP), and diet treatment*health status (interaction). For oral and I.V. plasma curves, 5 sampling timepoints, all two-way interactions, and the three-way interaction of health status*diet treatment*timepoint were performed. Posthoc group comparisons with Tukey adjustments were made as appropriate based on the overall model findings. Statistical significance was set at $\alpha<0.05$.

As BUN level is a biomarker of kidney function, BUN values were inspected for all rats. Sham rats with abnormal BUN values were suspected to be due to potential kidney injury during sham operation and were removed if they had a studentized deleted residual (SDR) ≥2.7. Outliers in BUN (n=5, 3 sham HP, 1 sham LP, and 1 CKD HP) were removed from all other analyses. n=1 rat (sham, LPHP) died at ~12 weeks of age, during the jugular catheter placement surgery despite no apparent complications; an enlarged stomach with compacted intestines were found upon necropsy. Thus, n=66 rats were included for analyses

of all outcomes of interest. For each outcome, outliers were investigated and removed if SDR was ≥ 3. Body weight and day-7 food intake were explored as additional covariates for intestinal fractional phosphorus absorption, but these were not significant and therefore, not included. Appropriate data transformations were performed for variables with non-normal distribution of residuals as determined by Shapiro-Wilk tests. Log-transformation was used for iFGF23, jejunal NaPi-2b, jejunal PiT1, and jejunal PiT2; and square-root transformation was used for analysis of duodenal NaPi-2b and PiT1. These variables were back-transformed and presented in the original units in the results. Results are reported as least-squares mean ± SD, unless otherwise indicated.

Results

During the 7-day diet study, rats progressively increased their daily food consumption in the 4-hour feeding window, with an average of 17.9 ± 4.42 g consumed on the final day. No difference was observed among groups for food consumption on day 7 (P=0.25) (**Table A.A.S2**). On day 7, sham rats had greater body weight compared with CKD rats (371 ± 27.3 g and 340 ± 27.2 g, respectively, P<0.0001). There was no difference in body weight by diet treatment nor an interaction effect (**Table A.A.S2**). As expected, the final plasma BUN was higher in CKD rats compared to sham rats (P<0.0001) (**Table 2.1**).

Intestinal fractional phosphorus absorption was not significant in the overall model (P=0.12) (**Table 2.1**). However, exploratory comparisons showed intestinal fractional phosphorus absorption efficiency tended to be higher in rats

in the LP group compared to rats in the HP group (*P*(diff)=0.053) or LPHP group (*P*(diff)= 0.054) (**Table 2.1, Figure 2.2A, Table A.A.S3**), whereas HP and LPHP groups were similar (*P*(diff)= 0.998). The absolute amount of dose absorbed was significantly higher in the HP (6.6 ± 2.2 mg P) and LPHP (6.7 ± 2.2 mg P) compared with the LP group (0.9 ± 2.2 mg P) (*P*(diff)<0.0001 for both diet comparisons) (**Figure 2.2B**). Additional detail on the oral and I.V. dose curves are shown in **Figure 2.3** and individual rat curves are provided in **Figure A.A.S1 and Figure A.A.S2**.

For plasma phosphorus, there was a significant health status by diet interaction (P<0.0001): Plasma phosphorus was not different between CKD and sham rats with the LP or HP diets, but with the LPHP diet, CKD rats had higher plasma phosphorus than sham rats (*P*(diff)=0.001). Regardless of health status, plasma phosphorus was higher with HP and LPHP diets compared to the LP diet (*P*(diff)<0.0001 for both comparisons, respectively) **(Table 2.1, Figure 2.2C, Table A.A.S3**).There was a significant main effect for diet on plasma calcium (*P*<0.0001): Plasma calcium values were significantly lower in rats in the LPHP group compared with rats in the HP group (*P*(diff)=0.006) and LP group (*P*(diff)<0.0001) but were not different between the HP and LP groups (*P*(diff)=0.56) (**Table 2.1, Table A.A.S3**).There was a significant health status by diet interaction for plasma iFGF23 (*P*=0.006). Plasma iFGF23 was lowest in rats in the LP group compared with the HP and LPHP groups (*P*(diff)<0.0001 for both comparisons, respectively), but there was only an effect of CKD within the HP

group where plasma iFGF23 was ~2x higher in CKD rats compared with sham (*P*(diff) <0.0001) (**Table 2.1, Figure 2.4A, Table A.A.S3**). There was a significant main effect for diet on plasma iPTH (*P*<0.0001), which was lowest in the LP group, followed by LPHP group and highest in the HP group (*P*(diff)<0.01 for all diet comparisons) (**Table 2.1, Figure 2.4B, Table A.A.S3**). The overall two-way ANCOVA model for 1,25D was not significant (*P*=0.53) (**Table 2.1, Figure 2.4C, Table A.A.S3**).

Intestinal phosphate transporters NaPi-2b, PiT-1, and PiT-2 were evaluated for changes in gene expression in the duodenum and jejunum. The overall two-way ANCOVA model for duodenal NaPi-2b and duodenal PiT-1 were not significant (*P*=0.27 and *P*=0.49, respectively) (**Table 2.2, Figure 2.5A and 2.5B, Table A.A.S4**). There was a significant diet effect for duodenal PiT-2 (*P*=0.0002). Duodenal PiT-2 mRNA expression was higher in rats in the LPHP group compared to rats in the LP and HP groups (*P*(diff)=0.0002 and *P*(diff)=0.02) (**Table 2.2, Figure 2.5C, Table A.A.S4**). There was a significant diet effect for jejunal NaPi-2b (*P*=0.01), where it was lower in rats in the LPHP group compared with rats in the HP group and tended to be lower than rats in the LP group(*P*(diff)=0.01 and *P*(diff)=0.06) (**Table 2.2, Figure 2.5D, Table A.A.S4**). The overall two-way ANCOVA model for jejunal PiT-1 was not significant (*P*=0.33) (**Table 2.2, Figure 2.5E, Table A.A.S4**). For jejunal PiT-2, the overall ANCOVA model was significant (*P*=0.0003), but this was only due to the

significant covariate (cohort), as there were no significant main effects or interaction (**Table 2.2, 2.5F, Table A.A.S4**).

Discussion

In the present study, we found that acute high phosphorus intake following a 6-day acclimation to a low phosphorus diet in 5/6 nephrectomized and sham-operated rats did not enhance intestinal fractional phosphorus absorption efficiency *in vivo*. This is supported by the lower (rather than higher) jejunal NaPi-2b mRNA expression observed in the LPHP group compared with the HP group. Despite this, the acute high phosphorus load in the LPHP group led to higher plasma phosphorus compared with the LP group but similar plasma phosphorus to that of the HP group. This was contrary to our hypothesis and the previous findings of Giral et al.[39] where plasma phosphorus was ~2 times higher in healthy rats fed an acute high phosphorus load following acclimation to a low phosphorus diet than rats only fed the high phosphorus diet chronically for all seven days (8.3±0.9 mg/dL vs 17.2±1.8 mg/dL). However, they studied the chronic high phosphorus diet and the low-to-acute high phosphorus diet in two separate experiments with slightly different feeding protocols (*ad libitum* vs 4-hour feeding window), which limits the comparison between these two treatments. In our study, we also observed a lower plasma Ca in the LPHP group but similar plasma Ca between the LP and HP groups. This could possibly be due to the rapid increase in phosphorus intake in LPHP rats when switched from the LP to

HP diet and an abrupt binding of dietary Ca leading to a transient decrease in serum Ca from lower Ca absorption.

In our study, CKD rats had higher iFGF23 compared with sham rats only when fed the HP diet for 7 days but was not different between CKD and sham rats in the LP group nor in the LPHP group that were both fed the low phosphorus diet for the first 6 days and only differed when the LPHP group was given the high phosphorus load on day 7. However, the acute high phosphorus load resulted in higher iFGF23 in both CKD and sham rats compared to CKD and sham rats kept on the low phosphorus diet. Others have observed increased iFGF23 in response to high phosphorus intakes[234–237] in humans, in line with its role as the major known phosphaturic hormone. But this has not been observed over a short time frame of only 4 hours[113], rather increases in iFGF23 have been seen as a delayed response after ~24 hours.[234–236] In our study, FGF23 was elevated within 6 hours of acute high phosphorus in the LPHP group. A possible explanation for this relatively quick response is that the acute high phosphorus diet was 10-fold higher in phosphorus content compared with the low phosphorus diet on which these rats were acclimated. This is consistent with the findings of Nishida et al.,[104] where FGF23 was measured at 4 and 8 hours after test meals of 400, 800, and 1200 mg P loads in non-CKD adults. FGF23 was only elevated at the 8-hour timepoint with the very high P load of 1200 mg. This indicates that higher dietary phosphorus may be required to observe a response in FGF23 at earlier timepoints. Notably, sham rats in the HP group fed the high phosphorus

diet for all seven days did not have higher iFGF23 than those in the LPHP group that only had 1 day of acute high phosphorus. Conversely, CKD rats fed the HP diet for 7 days had significantly higher plasma iFGF23 compared to those in the LPHP group, indicating that CKD rats may have a lower tolerance for sustained excess phosphorus.

There were no differences between CKD and sham rats for plasma 1,25D or iPTH. This is consistent with these hormones typically changing later in the disease progression compared with iFGF23, which is elevated as early as stage 2 CKD.[113,238] While numerous studies[59,61,239] have observed lower 1,25D in CKD rat models compared with controls, others[240] have reported no difference between 5/6th nephrectomized and control rats in a 9-week study. The diet effects on plasma iPTH, where the LP groups had the lowest values, followed by LPHP and highest with HP, are consistent with the expected effect on PTH as a phosphaturic hormone, increasing with higher dietary phosphorus exposure. Particularly, the higher iPTH in the LPHP group compared with the LP group is consistent with the observed role of PTH as an acute responder to alterations in dietary phosphorus intake.[101,104,241] Martin et al.[242] found that PTH increased as much as 80% within only 15 minutes of high phosphorus consumption in uremic rats.

The lack of enhanced phosphorus absorption efficiency in the LPHP group is contrary to previous findings in healthy rats that assessed intestinal phosphate uptake by *in vitro* methods.[39] Giral et al.[39] reported a five-fold higher sodium-

dependent uptake of phosphate in BBMV isolated from the duodenum of rats fed an acute high phosphorus load following acclimation to a low phosphorus diet. The differences in these findings may be attributable, at least in part, to the difference between the *in vivo* and *in vitro* techniques used to assess absorption efficiency in each study, in addition to the different rat models. The BBMV rapid filtration approach measures radioactive phosphorus uptake into BBMV isolated from intestinal mucosal scrapings, thus assesses transport of phosphate across the apical membrane but lacks the intracellular and basolateral membrane components of the cell. In contrast, the *in vivo* oral gavage intestinal absorption method used in this study keeps the intestinal epithelium intact and physiological systems in place.[243] Our results suggest that the enhanced *in vitro* phosphate uptake into isolated duodenal BBMV following an acute phosphorus load may not translate to enhanced *in vivo* intestinal phosphorus absorption efficiency along the entirety of the gastrointestinal tract. This adds to the few but growing number of *in vivo* intestinal phosphorus absorption studies contradicting previous findings from *ex vivo* methods of intestinal phosphate uptake. Although *ex vivo/in vitro* studies have repeatedly shown higher intestinal phosphate uptake efficiency induced by low phosphorus diets,[34,39,43,57,230,231,244,245] our group demonstrated that a low phosphorus diet (0.1% P w/w) did not affect intestinal phosphorus absorption efficiency in healthy male Sprague Dawley rats using a jejunal *in situ* ligated loop technique in live animals.[246] Marks et al.,[61] reported similar results using the same low phosphorus diet and jejunal ligated loop method for

assessing intestinal phosphorus absorption. In the current study, using the *in vivo* oral gavage test, we found only a non-significant trend towards enhanced intestinal phosphorus absorption efficiency with the LP diet.

With declining kidney function, rising FGF-23, and declining 1,25D, one would predict that intestinal phosphorus absorption efficiency would be suppressed. This is supported by studies showing that calcitriol increases expression of NaPi-2b and intestinal phosphorus uptake or transport as assessed by i*n vitro* and *ex vivo* methods in rodent models,[34,41,43–46] and conversely, vitamin D-receptor knock-out mice have lower expression of NaPi-2b.[58] However, *in vivo* absorption studies have shown a general lack of effect of kidney disease on intestinal phosphorus absorption efficiency. In the present study, this could be explained by the lack of reduced 1,25D in the CKD rats. However, lack of effect of kidney disease on *in vivo* intestinal phosphorus absorption efficiency has been demonstrated in three different rat models of CKD with decreased 1,25D: Marks et al.[61] found no difference in intestinal phosphorus absorption efficiency measured by the jejunal ligated loop method in 5/6th nephrectomized rats compared to sham. Turner et al.[62] reported similar results in an adenine-induced CKD rat model. And our group observed significantly *higher* intestinal phosphorus absorption efficiency in the Cy/+ rat model of progressive CKD-MBD compared to normal littermates, but the difference was only slight.[59] We also found that fractional intestinal phosphorus absorption, assessed by oral and I.V. administration of ^{33}P, was not different between patients with stage 3-4

CKD and healthy controls, despite lower 1,25D observed in the patients with CKD.[63] Thus, a compensatory response to decrease intestinal phosphorus absorption efficiency with declining kidney function appears to be lacking, at least in the moderate stages of disease progression. However, studies using *in vivo* intestinal phosphorus absorption methods are still relatively scarce; thus, more evidence is needed to draw firm conclusions regarding the discrepancies shown between the many *in vitro* studies and the few *in vivo* studies.

A rapid downregulation of jejunal NaPi-2b gene expression was observed for rats in the LPHP group compared to the HP group, which may partly explain the lack of enhanced intestinal fractional phosphorus absorption in the LPHP group compared to the enhanced uptake in BBMV of healthy male rats.[39] Similarly, Candeal et al.[230] observed lower duodenal and jejunal NaPi-2b and PiT-1 protein expression following acute high phosphorus intake compared to low phosphorus intake. However, the fractional absorption test methods used in this study give total fractional phosphorus absorption that includes both the active transcellular and passive paracellular absorption pathways. *In vivo,* transcellular phosphate absorption has been shown to account for ~1/3 of total phosphorus absorption.[30,59] Thus, paracellular phosphorus absorption likely contributes to the similar plasma phosphorus values between the LPHP and HP groups in this study. However, future studies are required to confirm this.

Fluctuating from a low phosphorus intake to an acute high phosphorus intake, as is often the case with CKD patients, does not subsequently enhance

fractional intestinal phosphorus absorption, but does lead to higher absolute phosphorus absorption and plasma phosphorus, according to our results. It also remains possible that other adverse consequences to such fluctuations may still occur. Indeed, Tani et al.[247] observed higher vascular calcification in unilateral nephrectomized male rats when given a diet fluctuating every two days from low phosphorus (0.02% P) to high phosphorus (1.2% P) diet over a 36 day period (averaging 0.6% P) compared to rats fed a consistent phosphorus diet of 0.6% P. The rats fed the fluctuating diet had similar vascular calcification compared with rats fed a consistent high phosphorus diet (1.2% P), despite the latter group consuming twice as much phosphorus over the course of the 36-day study. Similarly, Zelt et al.[248] observed greater phosphate and calcium accumulation in the vasculature following an acute I.V. phosphate pulse in male rats with adenine-induced CKD, suggesting that acute spikes in plasma phosphate may drive vascular calcification. This highlights the necessity of future studies investigating the effect of acute phosphorus intake following acclimation to a low phosphorus diet on cardiovascular endpoints in CKD.

One limitation of the current study was that intestinal phosphate transporter analysis was limited to mRNA expression, which may not accurately reflect changes in protein expression or location within the cytoplasm or brush border membrane. In addition, only a single bout of non-adherence to the low phosphorus diet was tested and may not represent intestinal fractional phosphorus absorption following multiple bouts of non-adherence or effects of

the diets over a longer duration. Phosphorus absorption measurement was limited to a 2-hour period, following the 4-hour feeding window. Therefore, we were unable to determine if enhanced phosphorus absorption occurred at an earlier time during this 4-hour window. If enhanced absorption took place, it is possible that compensation for the acute high phosphorus diet occurred by the time the phosphorus absorption test was performed. Further, the 2-hour absorption period only accounts for phosphorus absorption in the small intestine rather than the entirety of the gastrointestinal tract, which has been observed to take ~6 hours.[249] It is thought that the majority of phosphorus absorption occurs in the small intestine, although this remains uncertain.[31]

The low phosphorus diet level of 0.1% P w/w was chosen as consistent with low P diets commonly used in the rodent literature. However, this is only ~1/3 of the rat P requirement of 0.3% P w/w. Translating this to human intakes, this would be only ~210 mg/d P compared with the 700 mg/d P Recommended Dietary Allowance for adults.[153] Similarly, the 1.2% high P diet is 4x the rat requirement, which would translate to a human intake of ~2800 mg/d, which is higher than the estimated average U.S. intake (~1500 mg/d),[160] but still below the current Tolerable Upper Intake Level as set by National Academy of Medicine.[153] Thus, the difference in phosphorus intake between the LP and HP diets in this study is more drastic than what might typically be seen in humans switching from an unrestricted to a restricted diet. Yet, even with this relatively extreme dietary challenge, no effects on fractional phosphorus absorption were observed.

Differences in the main calcium sources in the two diets also existed, where the LP diet had calcium carbonate as the main calcium source, whereas the HP diet had calcium phosphate. Calcium carbonate would have a more metabolically alkaline effect compared with calcium phosphate, which could affect phosphorus handling at the intestine, kidney, and bone. In this study, only male rats were used, thus, we are unable to determine sex-differences in intestinal phosphorus absorption efficiency nor generalize our results to females. Moreover, the translation of findings from rodent models to humans must further be elucidated.[24] The major strength of this study was the use of the *in vivo* oral gavage technique to determine intestinal fractional phosphorus absorption efficiency, as this technique most accurately replicates physiological intestinal absorption compared to *ex vivo* or *in vitro* methods. Further, the incorporation of blinded randomization of rats to each group minimized the risk for bias.

Conclusion

In conclusion, the present study investigated how an acute high phosphorus load following a low phosphorus diet affected intestinal phosphorus absorption efficiency in 5/6 nephrectomized and sham-operated male rats using an *in vivo* oral gavage technique. Acute high phosphorus did not lead to enhanced fractional phosphorus absorption efficiency which provides evidence against the notion that dietary phosphorus restriction adversely increases absorption efficiency during dietary non-adherence, at least short-term. However, high phosphorus loads do result in greater absolute phosphorus absorption and

plasma phosphorus levels. Our data support continued efforts to limit phosphorus intake in patients with CKD. However, clinical studies are needed to confirm these findings in all stages of CKD and including cardiovascular endpoints.

Figure 2.1. Study design

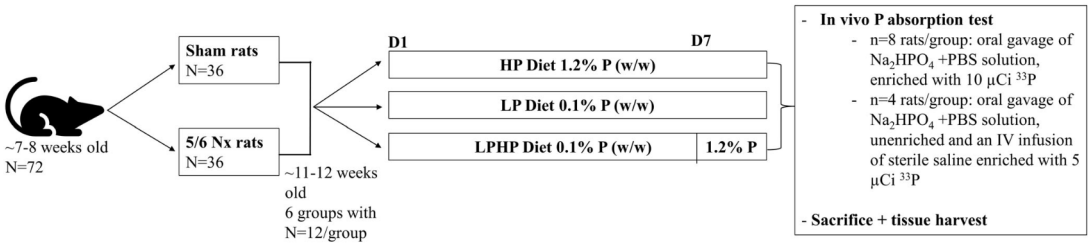


Figure 2.1. Study design

At ~7-8 weeks old, rats underwent either a two-step 5/6th nephrectomy or sham operation. Four weeks post-surgery (~11-12 weeks old), rats were switched to their randomly assigned diet treatment of either low phosphorus (LP, 0.1% w/w), high phosphorus (HP, 1.2% w/w), or low phosphorus followed by acute high phosphorus on the last day (LPHP, 0.1% w/w then 1.2% w/w). Rats were fed in a four-hour window (~8am-12pm) daily for seven days and received water *ad libitum*. Rats in the LPHP group were fed the LP diet on Days 1-6 and the HP diet on Day 7.

Table 2.1. Plasma biochemistries

	Health Status x Diet						ANCOVA P-Values			
	LP		LPHP		HP					
	Sham	CKD	Sham	CKD	Sham	CKD	Model	Health Status	Diet	HxD
Intestinal Fractional Phosphorus Absorption (AUC_{PO}/AUC_{IV})[1]	0.13 (0.05)	0.14 (0.05)	0.08 (0.05)	0.10 (0.06)	0.08 (0.05)	0.10 (0.06)	0.12	0.31	**0.03**	0.97
BUN (mg/dL)[2]	23.3 (5.11)	40.3 (5.09)	21.9 (5.11)	39.3 (5.09)	22.2 (5.16)	37.2 (5.11)	**<0.0001**	**<0.0001**	0.40	0.73
P (mg/dL)[3]	6.3 (1.13) c	5.2 (1.14) c	11.4 (1.13) b	13.5 (1.13) a	11.4 (1.14) b	12.5 (1.14) a,b	**<0.0001**	**0.02**	**<0.0001**	**<0.0001**
Ca (mg/dL)[4]	11.3 (1.16)	11.4 (1.14)	10.3 (1.14)	9.3 (1.14)	10.7 (1.14)	11.2 (1.16)	**<0.0001**	0.71	**0.0001**	0.08
iFGF23 (pg/mL)[5]	126 (46.1) c	183 (63.4) c	340 (118.6) b	420 (145.8) b	327 (117.9) b	837 (302.1) a	**<0.0001**	**<0.0001**	**<0.0001**	**0.006**
iPTH (pg/dL)[6]	820 (633.8)	594 (632.5)	1559 (633.8)	1114 (632.5)	1990 (637.5)	2358 (633.8)	**<0.0001**	0.53	**<0.0001**	0.12
$1,25(OH)_2D_3$ (pg/mL)[7]	143 (50.4)	133 (50.1)	157 (50.3)	142 (49.9)	142 (50.4)	161 (50.1)	0.53	0.87	0.68	0.51

Table 2.1. Plasma biochemistries

ANCOVA p-values for the overall model (P_{Model}), main effect of health status (P_{Health}), diet (P_{Diet}), and their interaction (P_{HxD}) for intestinal fractional phosphorus absorption and plasma biochemistires. LS means ± SD are shown. Intestinal fractional phosphorus absorption was not different between groups. Plasma BUN was higher in CKD rats compared to sham. Plasma phosphorus was higher in HP and LPHP groups compared to the LP group regardless of health status and was higher in CKD rats in the LPHP group compared to sham rats in the LPHP group. Plasma calcium was lower in the LPHP group compared to rats in the HP and LP group. Plasma iFGF23 was lowest in rats in the LP group and was highest in CKD rats in the HP group. Plasma iPTH was lowest in rats in the LP group followed by rats in the LPHP group, and highest in rats in the HP group.

Plasma 1,25D did not differ between groups. Different superscripted letters represent group differences.

[1]n= 7 sham LP, 7 sham LPHP, 6 sham HP, 7 CKD LP, 8 CKD LPHP, 8 CKD HP

[2]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

[3]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 11 CKD LPHP, 10 CKD HP

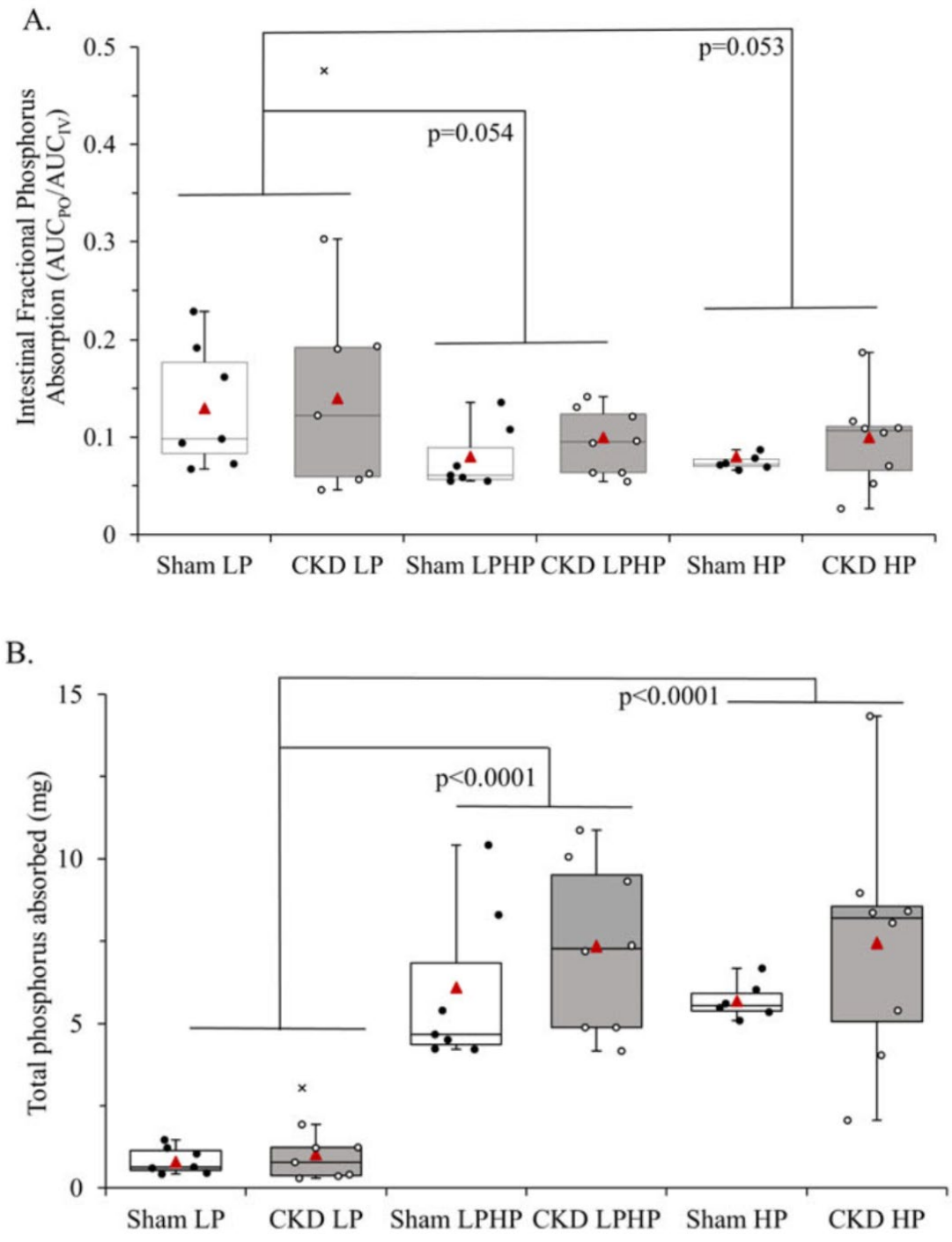
[4]n= 11 sham LP, 10 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

[5]n= 11 sham LP, 10 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 9 CKD HP

[6]n= 11 sham LP, 11 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

[7]n= 9 sham LP, 10 sham LPHP, 9 sham HP, 11 CKD LP, 12 CKD LPHP, 11 CKD HP

Figure 2.2. Phosphorus absorption and plasma phosphorus



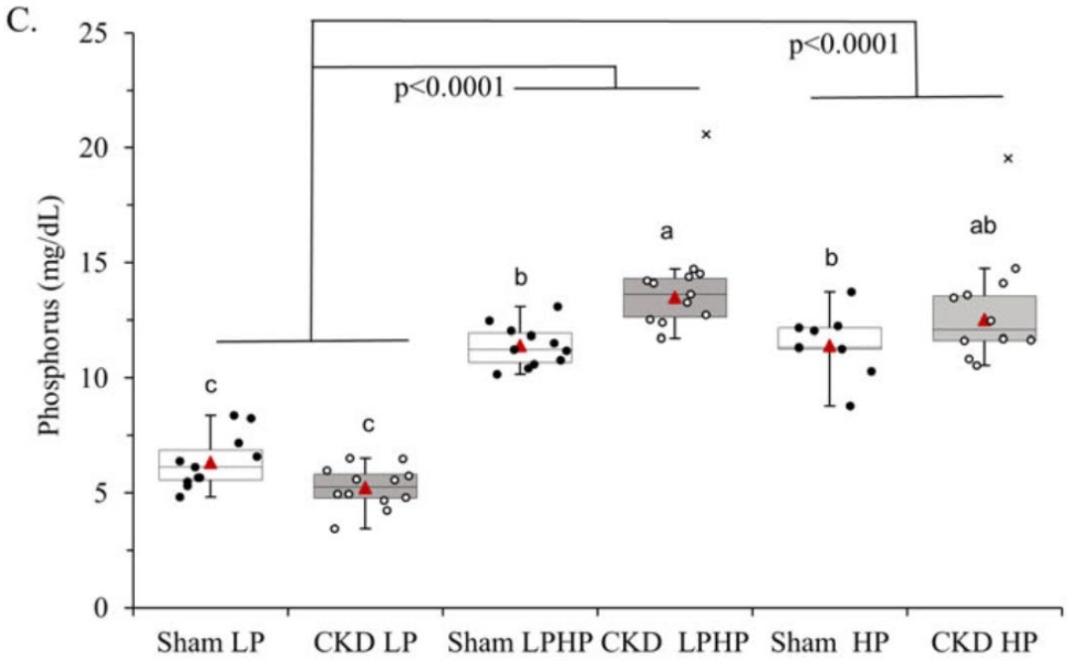


Figure 2.2. Phosphorus absorption and plasma phosphorus
A) Intestinal fractional phosphorus absorption by health status and dietary phosphorus load. Intestinal fractional phosphorus absorption was determined from the ratio of area under the oral and I.V. plasma ^{33}P curves (AUC_{PO}/AUC_{IV}) over a 2-hour period. Intestinal fractional phosphorus absorption was not significant in the overall model ($P_{Model} = 0.12$, $P_{Health} = 0.31$, $P_{Diet} = 0.03$, $P_{HxD} = 0.97$). **B) Absolute amount of phosphorus absorbed by health status and dietary phosphorus load.** Rats in the HP and LPHP group absorbed a significantly greater amount of the dose compared with the LP group ($P_{Model} < 0.0001$, $P_{Health} = 0.12$, $P_{Diet} < 0.0001$, P_{HxD} 0.65). **C) Plasma phosphorus by health status and dietary phosphorus load.** CKD and sham rats in the LPHP and HP groups had higher plasma phosphorus than rats in the LP group. Within

diets, CKD rats had higher plasma phosphorus compared to sham only in the LPHP group (P_{Model} < 0.0001, P_{Health}= 0.02, P_{Diet} < 0.0001, P_{HxD} < 0.0001*).* The median, interquartile range, minimum, and maximum, including outliers are shown. “x” symbols denote outliers not included in ANCOVA. LS means are represented by triangles for each group. Sham rats are shown with white boxes and CKD rats are shown with grey boxes.

LP= low phosphorus diet, HP = high phosphorus diet, LPHP = low phosphorus for 6 days followed by acute high phosphorus on day 7.

Fractional phosphorus absorption and amount of dose absorbed n = 7 sham LP, 7 sham LPHP, 6 sham HP, 7 CKD LP, 8 CKD LPHP, 8 CKD HP

Plasma phosphorus n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Figure 2.3. Average oral and I.V. dose curves

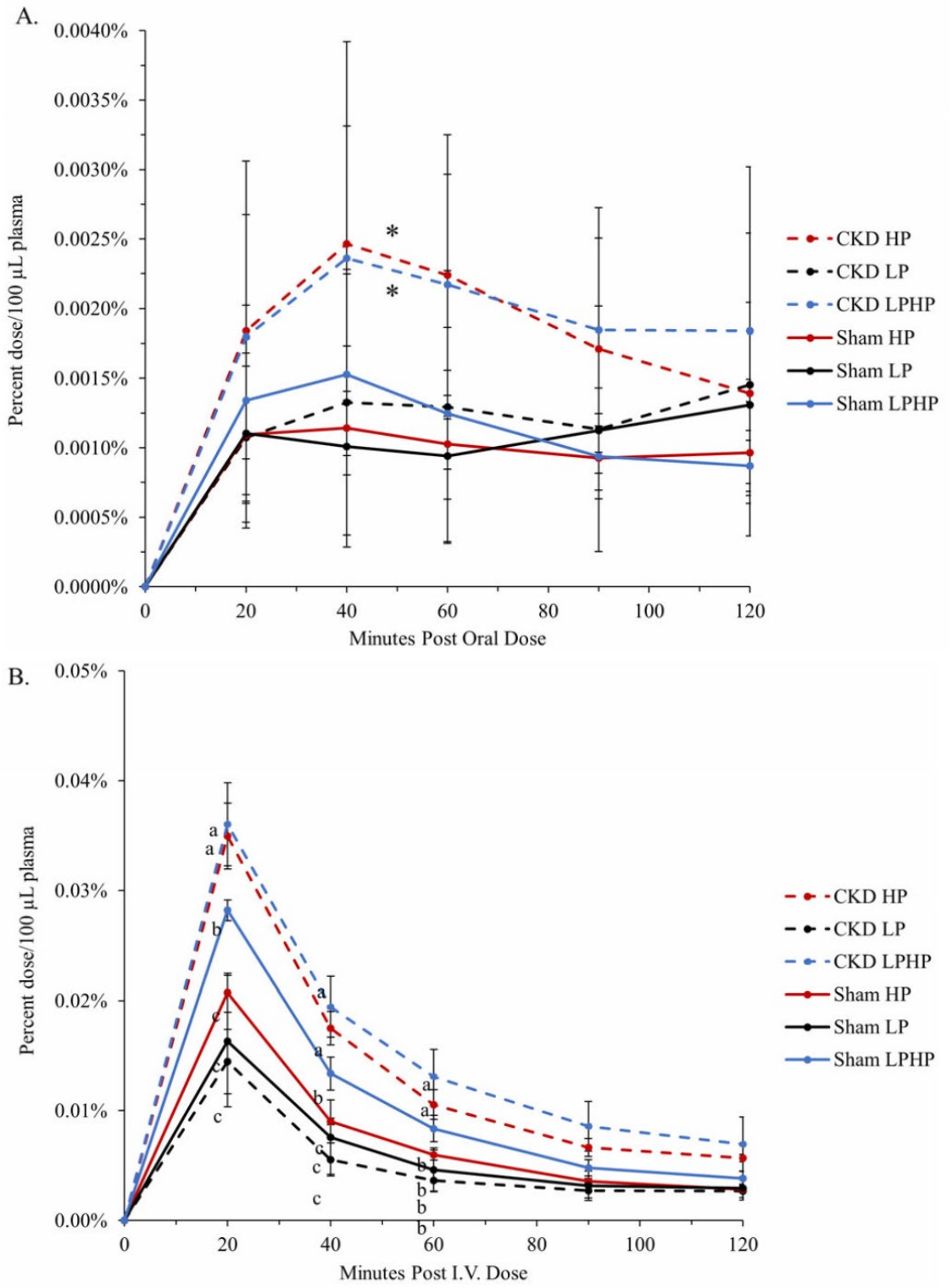
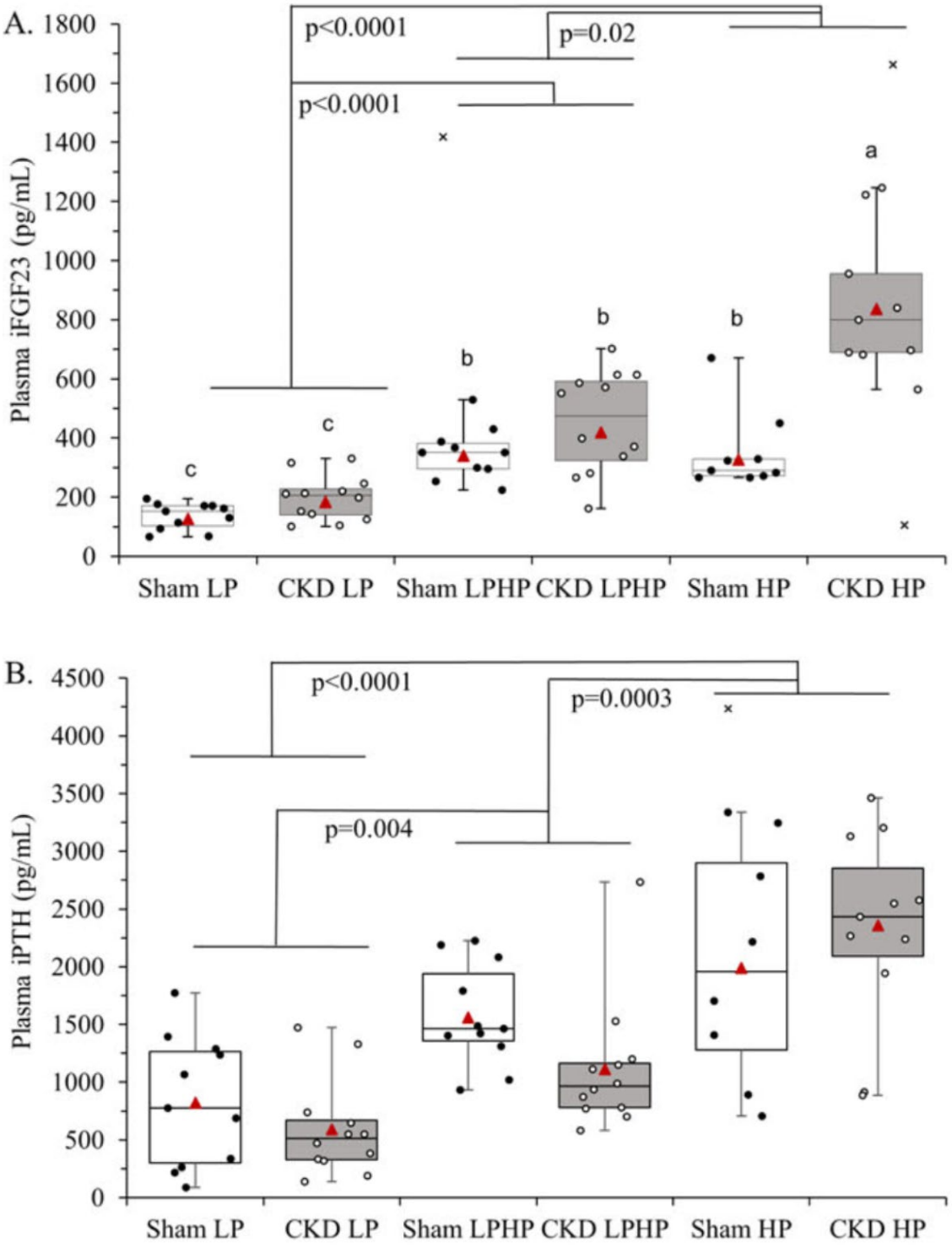


Figure 2.3. Average oral and I.V. dose curves
A) Average oral dose curves. Group mean plasma ^{33}P levels at each timepoint are shown as percent of oral isotope dose with SD error bars. No difference was observed for the interaction of health status by diet treatment by timepoint over the 2-hour period (*P*=0.98). There was significant two-way interaction between diet treatment and health status (P=0.03). *Mean plasma ^{33}P as percent of oral isotope dose was significantly higher in the CKD HP and CKD LPHP groups compared with all other group means over the 120 min period (P<0.05), but there were no significant differences at any individual timepoint. **B) Average I.V. dose curves**. Group mean plasma ^{33}P levels at each timepoint are shown as percent of I.V. isotope dose with SD error bars. There was a significant three-way interaction of health status by diet treatment by timepoint (*P*=0.007). Posthoc comparisons of group means within each timepoint were evaluated. Within each timepoint, group means that are significantly different are indicated by different letters ($p < 0.05$). Mean plasma 33P as percent of I.V. isotope dose was significantly higher in the CKD HP and CKD LPHP groups at 20-, 40-, and 60-minutes compared with all other groups. Additionally, the sham LPHP group had higher plasma 33P compared with the sham LP, sham HP diets CKD LP groups at 20- and 40- minutes. By 90 and 120 minutes there were no differences between groups. Values presented are from 100 µL of plasma counted at each time point over a 2-hour period.

Average oral dose curves: n= 7 sham LP, 7 sham LPHP, 6 sham HP, 7 CKD LP, 8 CKD LPHP, 8 CKD HP

Average I.V. dose curves: n= 4 sham LP, 4 sham LPHP, 3 sham HP, 4 CKD LP, 4 CKD LPHP, 3 CKD HP

Figure 2.4. Plasma FGF-23, PTH, and 1,25D



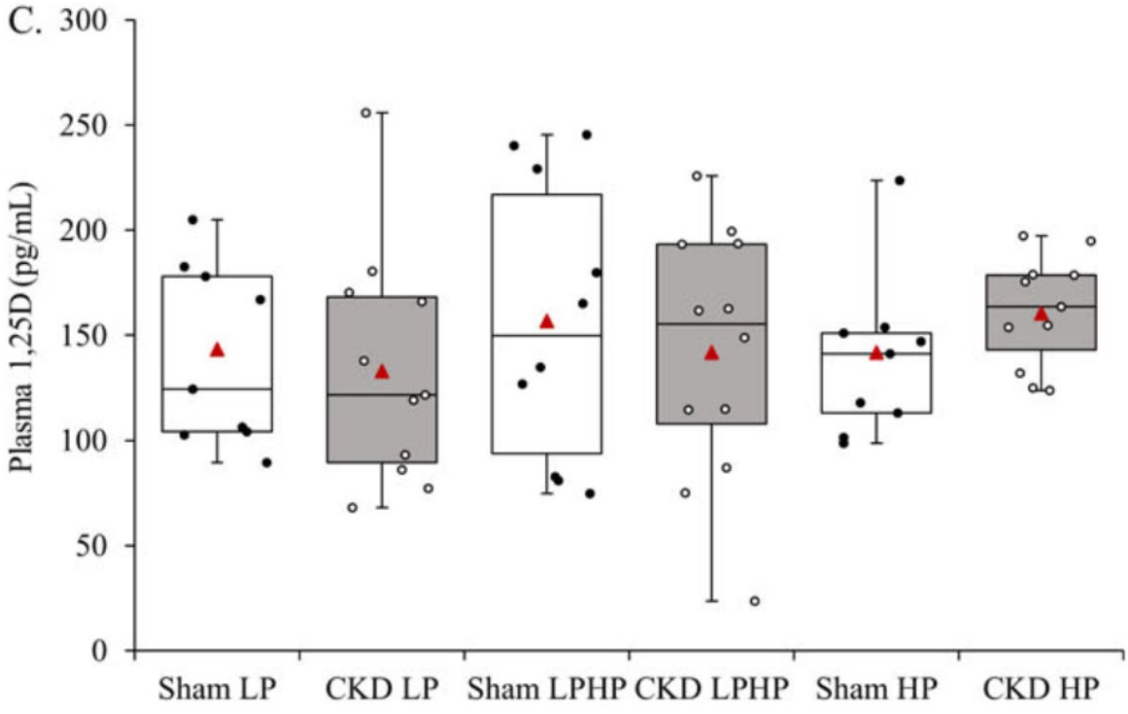


Figure 2.4. Plasma FGF-23, PTH, and 1,25D
A) Plasma iFGF23 by health status and dietary phosphorus load. Plasma iFGF23 was lowest in rats in the LP group regardless of health status and was ~2x higher in CKD rats in the HP group compared to any other group (P_{Model} < 0.0001, P_{Health} < 0.0001, P_{Diet} < 0.0001, P_{HxD} = 0.006). **B) Plasma iPTH by health status and dietary phosphorus load.** Plasma phosphorus was lowest in rats in the LP group, followed by the LPHP group, and highest in the HP group (P_{Model} < 0.0001, P_{Health} = 0.53, P_{Diet} < 0.0001, P_{HxD} = 0.12). **C) Plasma 1,25D by health status and dietary phosphorus load.** Plasma 1,25D did not differ between groups (P_{Model} = 0.53, P_{Health} = 0.87, P_{Diet} = 0.68, P_{HxD} = 0.51). The median, interquartile range, minimum, and maximum, including outliers are shown. “x”

symbols denote outliers not included in ANCOVA. LS means are represented by triangles for each group. Sham rats are shown with white boxes and CKD rats are shown with grey boxes.

LP= low phosphorus diet, HP = high phosphorus diet, LPHP = low phosphorus for 6 days followed by acute high phosphorus on day 7.

Plasma iFGF23 and iPTH n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Plasma 1,25D n = 9 sham LP, 10 sham LPHP, 9 sham HP, 11 CKD LP, 12 CKD LPHP, 11 CKD HP

Table 2.2. Gene expression of intestinal phosphate transporters

	Health Status x Diet						ANCOVA P-Values			
	LP		LPHP		HP					
	Sham	CKD	Sham	CKD	Sham	CKD	Model	Health Status	Diet	HxD
Duodenal NaPi-2b/RPLP0[1]	2.50 (1.89)	1.80 (1.59)	1.37 (1.39)	2.28 (1.79)	2.59 (1.92)	1.82 (1.63)	0.27	0.72	0.71	0.17
Duodenal Pit-1/RPLP0[2]	1.23 (0.66)	0.83 (0.52)	0.83 (0.53)	1.25 (0.62)	1.25 (0.66)	1.02 (0.57)	0.49	0.66	0.82	0.0501
Duodenal Pit-2/RPLP0[1]	0.88 (0.36)	0.93 (0.35)	1.33 (0.36)	1.42 (0.36)	1.12 (0.36)	1.01 (0.36)	**0.01**	0.91	**0.0002**	0.64
Jejunal NaPi-2b/RPLP0[3]	4.26 (4.24)	1.99 (2.02)	1.36 (1.39)	1.55 (1.52)	3.03 (3.05)	4.22 (4.28)	**0.007**	0.69	**0.01**	0.18
Jejunal Pit-1/RPLP0[4]	3.49 (3)	2.27 (1.97)	1.77 (1.59)	2.48 (2.18)	2.18 (1.95)	3.82 (3.32)	0.33	0.47	0.41	0.16
Jejunum Pit-2/RPLP0[5]	1.58 (0.63)	1.35 (0.55)	1.65 (0.70)	1.46 (0.62)	1.55 (0.65)	1.36 (0.53)	**0.0003**	0.19	0.86	0.98

Table 2.2. Gene expression of intestinal phosphate transporters

ANCOVA p-values for the overall model (P_{Model}), main effect of health status (P_{Health}), diet (P_{Diet}), and their interaction (P_{HxD}) for the intestinal phosphate transporters NaPi-2b, PiT-1, and PiT-2. LS means ± SD are shown. Duodenal NaPi-2b and PiT-1 mRNA expression did not differ between groups. Duodenal PiT-2 mRNA expression was higher in rats in the LPHP group compared to rats in the LP and HP groups. Jejunal NaPi-2b mRNA expression was lower in rats in the LPHP group compared to rats in the HP group and tended to be lower than rats in the LP group. Jejunal PiT-1 and PiT-2 mRNA expression did not differ between groups.

[1]n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 11 CKD LPHP, 11 CKD HP

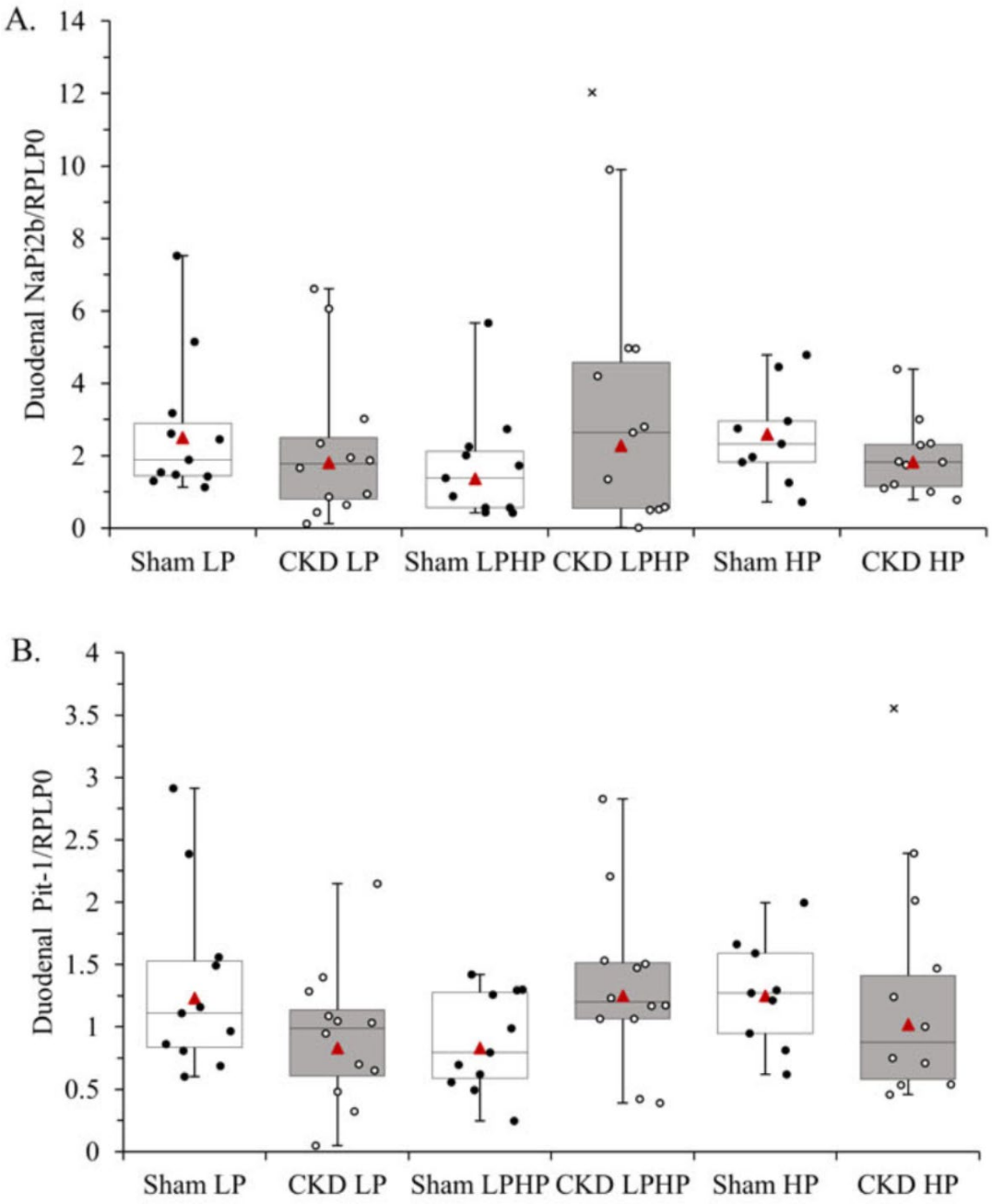
[2]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 10 CKD HP

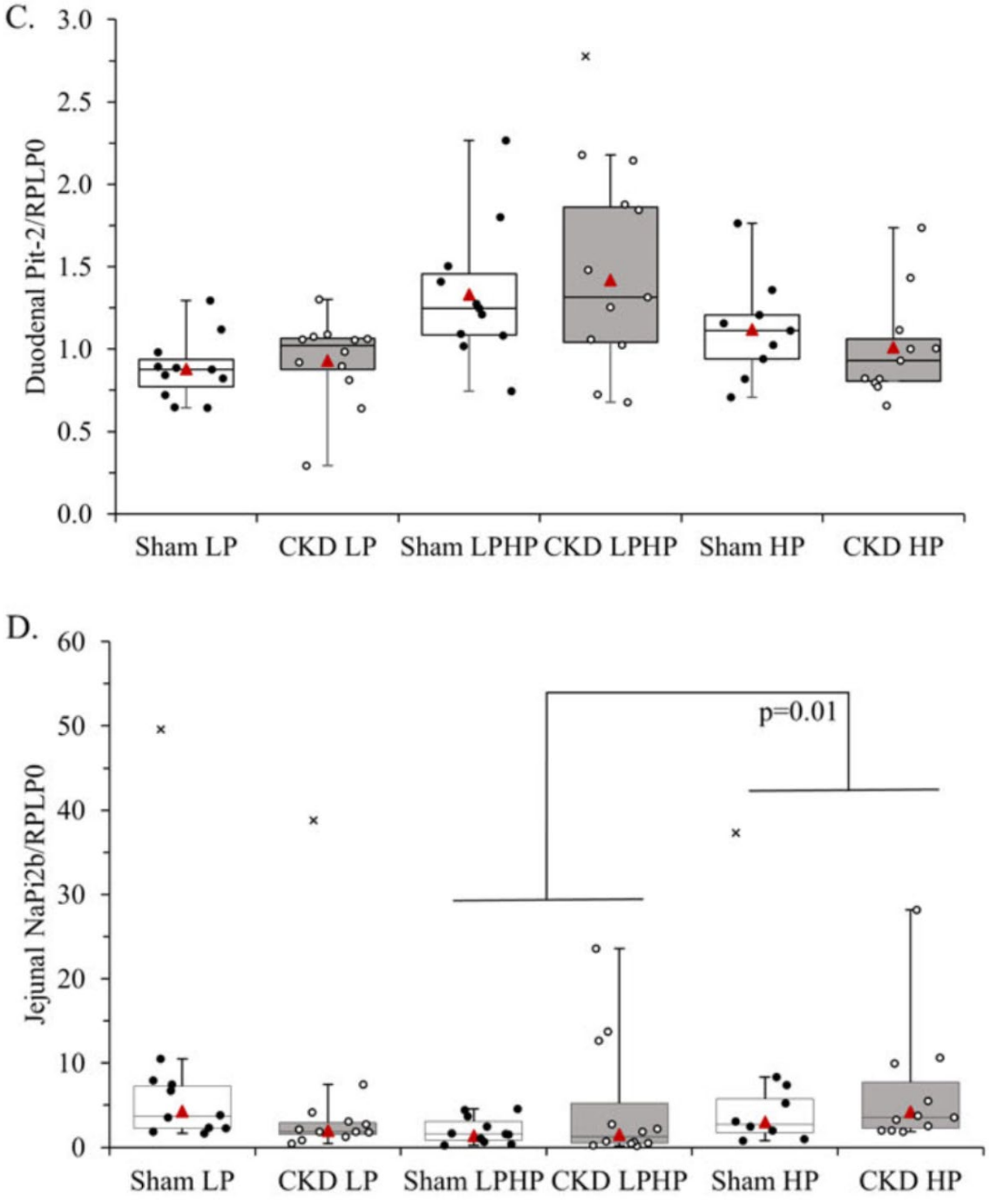
[3]n= 10 sham LP, 11 sham LPHP, 8 sham HP, 11 CKD LP, 12 CKD LPHP, 11 CKD HP

[4]n= 10 sham LP, 10 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

[5]n= 11 sham LP, 10 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Figure 2.5. Duodenal and jejunal phosphate transporters




C.
Duodenal Pit-2/RPLP0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sham LP
CKD LP
Sham LPHP
CKD LPHP
Sham HP
CKD HP
D.
Jejunal NaPi2b/RPLP0
60
50
40
30
20
10
0
p=0.01
Sham LP
CKD LP
Sham LPHP
CKD LPHP
Sham HP
CKD HP

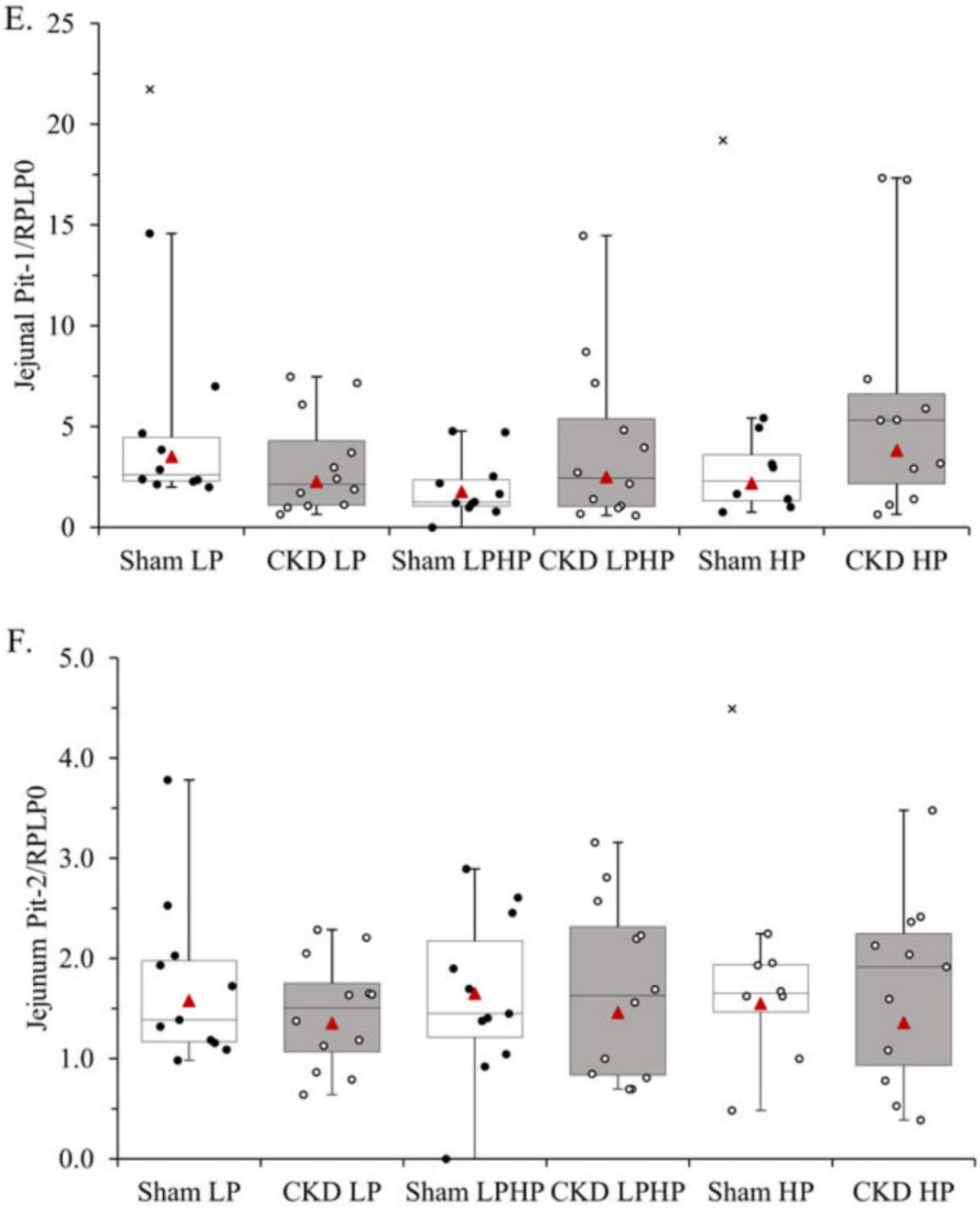


Figure 2.5. Duodenal and jejunal phosphate transporters
A) Duodenal NaPi-2b mRNA expression by health status and dietary phosphorus load. Duodenal NaPi-2b mRNA expression did not differ between groups (P_{Model}=0.27, P_{Health} = 0.72, P_{Diet} = 0.71, P_{HxD} = 0.17). **B) Duodenal PiT-1**

mRNA expression by health status and dietary phosphorus load. Duodenal PiT-1 mRNA expression did not differ between groups (P_{Model} = 0.49, P_{Health} = 0.66, P_{Diet} = 0.82, P_{HxD} = 0.0501). **C) Duodenal PiT-2 mRNA expression by health status and dietary phosphorus load.** Duodenal PiT-2 mRNA expression was higher in rats in the LPHP group compared to rats in the LP and HP groups (P_{Model} = 0.01, P_{Health} = 0.91, P_{Diet} = 0.0002, P_{HxD} = 0.64). **D) Jejunal NaPi-2b mRNA expression by health status and dietary phosphorus load.** Jejunal NaPi-2b mRNA expression was lower in rats in the LPHP group compared to rats in the HP group and tended to be lower than rats in the LP group (P_{Model} = 0.007, P_{Health} = 0.69, P_{Diet} = 0.01, P_{HxD} = 0.18). **E) Jejunal PiT-1 mRNA expression by health status and dietary phosphorus load.** Jejunal PiT-1 mRNA expression did not differ between groups (P_{Model} = 0.33, P_{Health} = 0.47, P_{Diet} = 0.41, P_{HxD} = 0.16). **F) Jejunal PiT2 mRNA expression by health status and dietary phosphorus load.** Jejunal PiT-2 mRNA expression did not differ between groups (P_{Model} = 0.0003, P_{Health} = 0.19, P_{Diet} = 0.86, P_{HxD} = 0.98). The significance in the P_{Model} is due to a cohort effect rather than main effects for health status, diet, or their interaction. Expression was calculated relative to Rplp0. The median, interquartile range, minimum, and maximum, including outliers are shown. “x” symbols denote outliers not included in the ANCOVA. LS means are represented by triangles for each group. Sham rats are shown with white boxes and CKD rats are shown with grey boxes.

LP= low phosphorus diet, HP = high phosphorus diet, LPHP = low phosphorus for 6 days followed by acute high phosphorus on day 7.

Duodenal NaPi-2b, duodenal PiT1, and duodenal PiT2 n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Jejunal Napi-2b, jejunal PiT1 and jejunal PiT2 n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Chapter 3: Phosphorus Bioaccessibilty of Emerging Processed Pulse and Soy-Based Protein Products by *In Vitro* Simulation of Human Digestion

Abstract

Consumption of plant-based protein for the dietary management of phosphorus in CKD is of growing interest due to several proposed benefits, including potentially lower phosphorus bioaccessibility. However, data on phosphorus bioaccessibility are generally lacking, particularly for new and emerging plant-based protein products. Therefore, this pilot study aimed to compare phosphorus bioaccessibility of emerging plant-based protein products to their animal protein counterparts using *in vitro* simulation of human digestion.

Soy-based (n=16), pulse-based (n=17), and animal-based (n=13) products from 4 different food categories (beef, dairy, sausage/bacon, chicken/turkey) were evaluated. Products were prepared according to package directions and freeze dried prior to *in vitro* digestion experiments which consisted of an oral, gastric, and intestinal phase, then final digesta were dialyzed for 30 hours. Phosphorus content was analyzed in the pre-digestion samples, final digesta, and final dialysate samples by MP-AES to calculate total and bioaccessible phosphorus.

Average percent bioaccessible phosphorus of plant-based products ranged from 32-103% while animal products ranged from 81-110%. Absolute bioaccessible phosphorus was lower for soy- and pulse-based beef and

chicken/turkey, and pulse-based sausage/bacon compared to their animal counterpart products. Pulse-based milk had ~2.5x the average absolute bioaccessible phosphorus than soy-based and cow's milk. Additionally, soy-based milk had the lowest percent bioaccessible phosphorus compared with pulse-based and cow's milk, despite the presence of at least one inorganic phosphate-containing additive on the ingredient list.

Most soy- and pulse-based-protein products offered lower bioaccessible phosphorus per 100g serving than their animal counterparts. Thus, this pilot investigation indicates that certain plant-based protein products may be suitable for inclusion in the diets of patients with CKD, but more data on phosphorus bioaccessibility is needed to better inform individualized guidance.

Introduction

Demand for plant-based protein alternatives to animal sources has led to dramatic growth in the development and availability of processed plant-based protein products. Sales for plant-based protein products have increased 54% in the past three years with plant-based milk alternatives, other plant-based dairy alternatives, and plant-based meat alternatives making up a majority of sales ($2.6, $2.1, and $1.4 billion, respectively).[250] Likewise, interest in plant-based protein for dietary management of chronic kidney disease (CKD) continues to grow as there are several proposed benefits[251,252], including potentially lower phosphorus bioaccessibility.[136] Phosphorus 'bioaccessibility' refers to the amount of phosphorus digested and accessible to be absorbed whereas 'bioavailability' refers to the amount of phosphorus digested, absorbed, and available for use by the body.[203,253] Based on promising but still very limited data, both the 2020 Kidney Disease Outcomes Quality Initiative (KDOQI) Nutrition in CKD 2020 Update and Kidney Disease Improving Global Outcomes (KDIGO) 2017 CKD-Mineral and Bone Disorder (CKD-MBD) Update guidelines suggest considering phosphorus source (animal, plant, or additive) when making dietary recommendations for phosphorus management in patients with CKD.[121,254]

Estimates of phosphorus bioaccessibility range from ~ 10-30%[22] to ~40-50%[136] for plant sources, ~40-60% for animal sources[22], and up to ~100% for inorganic phosphate-containing food additives.[22] Lower bioaccessibility of phosphorus from plant sources is largely attributable to its form as phytic acid,

which accounts for ~50-82% of total phosphorus in plants;[210,211] humans do not produce the phytase enzyme and have limited capacity to hydrolyze phytic acid to liberate phosphate in the gastrointestinal tract.[215,255] Lower phosphorus bioaccessibility from plant sources may offer an advantage for dietary choices to reduce overall phosphorus burden. However, few studies[216–218] are available that have directly measured phosphorus bioaccessibility by *in vitro* simulation of human digestion. These studies provide valuable data on phosphorus bioaccessibility on a variety of food products that generally substantiate estimates for percent phosphorus bioaccessibility from plant, animal, and inorganic sources of phosphorus, though with wide ranges: ~6-42% from legume and seed sources,[216] 29-99% from grain sources,[216,218] ~70-107% from meat sources,[217] ~49-111% from dairy sources,[217] and 84-100% from inorganic sources.[216] Importantly, the small number of plant protein foods included were limited to raw/uncooked products, and the studies were conducted over a decade ago, thus, they naturally did not include processed plant-based protein products that have emerged in recent years. Various processing and cooking methods can affect phosphorus content of animal and plant protein foods and could affect non-enzymatic hydrolysis of phytic acid in plant foods.[256–259] increasing phosphorus bioaccessibility. But data on phosphorus bioaccessibility of emerging processed plant protein products is absent from the literature.

In addition to the guideline statements regarding phosphorus food source, the KDOQI 2020 nutrition guidelines[121] provide additional commentary that

healthcare practitioners should “advise choosing natural foods that are lower in bioavailable phosphorus” and “advise choosing commercial food items prepared without phosphorus-containing food additives.” This is based on the premise that more highly processed foods would have undesirable qualities (e.g., higher sodium, higher fat, added sugar, lower fiber) including higher inorganic-phosphate containing food additives. Without the necessary data for more precise and nuanced guidance, this commonsense approach is prudent. However, processed foods can also confer benefits that may include convenience, shelf-stability, texture, flavor, and other sensory attributes that have potential to improve nutrition, independence, and quality of life for individuals with diverse needs.[260,261] Therefore, the aim of this pilot study was to determine phosphorus bioaccessibility of emerging processed plant-based protein products (soy and pulse-based) in comparison to their animal protein counterpart products using *in vitro* methods of simulated human digestion.

Methods

Product Procurement and Preparation

Pulse-based protein, soy-based protein, and animal protein counterpart products were selected for this study. Products were chosen based on popular demand in the United States based on retail sales data from the Good Foods Institute and SPINS data[250] and by sorting products online using “best seller” filters. Products were selected in the following categories: beef, dairy (milk, cheese, and yogurt), chicken and turkey, and sausage and bacon. Forty-six total

products including 17 pulse-based, 16 soy-based and 13 animal-based proteins were included. All products were purchased from nationwide retailers located in the Minneapolis-St. Paul metro area of Minnesota (United States) between August 2021-January 2022. Natural or traditional forms of plant protein products (e.g., tofu) (n=17) were also evaluated for comparison. Information on phosphate containing food additives was obtained from the ingredient list on each product package (**Table A.C.S1**).

Products were prepared according to package directions which included methods such as cooking in an oven or microwave, or on a skillet. Dried legume products were prepared by soaking then subsequent boiling (**Table A.C.S2**). Tap water was used in the cooking process when necessary. No additional oils, spices, or ingredients were used. Prepared products were frozen at -20°C for at least 24 hours then freeze dried (FTS systems, Dura-Dry Condense Module Bulk Tray Dryer) for 4-7 days. Following freeze drying, products were pulverized into small particles (~2.2mm or less) using a mallet and mortar and pestle. All freeze-dried samples were kept at room temperature until use.

Total Phosphorus

Total phosphorus was determined from the freeze-dried food samples (pre-digestion). Five replicates of each freeze-dried food sample were dry-ashed in a muffle furnace (Thermo Scientific, Thermolyne Furnace, Atmosphere Controlled Ashing, Model F30400). Briefly, 2-3g of freeze-dried food sample were weighed into porcelain crucibles and placed in a muffle furnace. Samples were

heated to 300°C at a rate of 3°C per minute and held at 300°C for 16 hours, then ramped to 600°C at a rate of 3°C per minute and held at 600°C for 72 hours. Ashed sample was dissolved with 1 mL of concentrated nitric acid and diluted with ultrapure water to 25 mL. Samples were filtered using a 0.45µm filter to remove particulates and then analyzed for phosphorus by microwave plasma atomic emission spectroscopy (MP-AES).

In Vitro Digestion Experiments

Reagent Preparation

Simulated salivary, gastric and intestinal fluids were prepared to closely mimic the composition of human digestive fluids according to the INFOGEST protocol recommendations[262] and consisted of the following electrolyte solutions: 0.5M potassium chloride, 0.5M monopotassium phosphate, 1M sodium bicarbonate, 2M sodium chloride, 0.15M magnesium chloride hexahydrate, 0.5M ammonium carbonate, and 6M hydrochloric acid with the exception that the simulated salivary fluid did not include sodium chloride and the simulated intestinal fluid did not include ammonium carbonate. Stock solutions of each simulated fluid were prepared at a 1.25x concentration and were kept at -20°C until use. Prior to use in the *in vitro* digestion experiments, simulated fluids were prewarmed to 37°C in a shaking water bath.

All enzyme solutions were prepared the day of the *in vitro* digestion experiments. α-amylase (from *bacillus sp.,* Sigma Aldrich A6380) and pepsin (from porcine gastric mucosa, Sigma Aldrich P7012) solutions were prepared

with ultrapure water, and pancreatin (from porcine pancreas, Sigma Aldrich P7545) and bile (porcine, Sigma Aldrich B8631) solutions were prepared with simulated intestinal fluids. The concentration of the prepared α-amylase solution was 75 units per mL and pepsin solution was 2000 units/mL. Units of activity for amylase and pepsin were determined according to the manufacturer certificate of analysis. The concentration of bile used was 40 mg/mL and the concentration of pancreatin used was normalized to the amount of trypsin activity, 100 units/mL. The trypsin activity of pancreatin was determined based on the method described in the INFOGEST protocol.[262] Briefly, 2.6 mL of a 46 mM Tris/HCl buffer and 0.3 mL of 10 mM na-tosyl-arginine-methyl-ester were pipetted into a cuvette and warmed to 25°C. Pancreatin was added to the cuvette in varying concentrations 0.1, 0.5, 0.75, 1 and 1.5 mg/mL, and the absorbance increase at 247 nm was measured by UV-vis spectrometry and recorded in continuum over 10 minutes, until levelling off.

In Vitro Digestion Protocol

Freeze dried food products were digested by *in vitro* simulation of human digestion according to the standardized INFOGEST protocol[262] with modifications to determine phosphorus bioaccessibility (**Figure 3.1**). The *in vitro* experiments consisted of an oral, gastric, and intestinal phase to mimic the physiology of human digestion. Each sample was digested in triplicate. In addition, a 'process blank' sample, using ultrapure water, was prepared during the *in vitro* digestion experiments to determine background phosphorus. Each food product was pre-

tested to determine adjustments needed by HCl and NaOH to achieve target pH levels of the three simulated digestion phases (**Appendix C**). For the oral phase, one gram of freeze-dried food sample or ultrapure water (blank) was weighed into a 50 mL falcon tube. Then 0.8 mL of simulated salivary fluid, 0.1 mL of α-amylase, 0.005 mL of 0.3 M calcium chloride ($CaCl_2$), and 1.095 mL of ultrapure water were added to each tube. The final volume of the oral phase was 3 mL. Following this, the tubes were placed in a shaking water bath with a temperature of 37°C and oscillated at 110-115 oscillations per minute (OPM) for 2 minutes. For the gastric phase, 2.4 mL of simulated gastric fluid, 0.15 mL of pepsin, and 0.00015 mL of 0.3 M $CaCl_2$ was added to each tube, and pH adjusted to 3. Ultrapure water was added to achieve a final gastric phase volume of 6 mL. Tubes were then placed back in the shaking water bath (37°C and 110-115 OPM) for 2 hours. After 2 hours, 2.55 mL of simulated intestinal fluid, 1.5 mL of pancreatin, 0.75 mL of bile, and 0.012 mL of 0.3 M $CaCl_2$ was added to start the intestinal phase. The pH was adjusted to achieve a pH of 7 and ultrapure water was added to reach a final intestinal phase volume of 12 mL. The tubes were again incubated in the shaking water bath (37°C and 110-115 OPM) for 2 hours. Upon completion of the intestinal phase, the final digesta was diluted with ultrapure water to 25 mL.

Bioaccessible Phosphorus

Following the *in vitro* digestion experiments, 5 mL of diluted digesta of each sample and the blank were pipetted into a 500-1000 Dalton molecular

weight cut off dialysis membrane device (float-a-lyzer® G2, Repligen G235051) and dialyzed against 40 mL of ultrapure water for 30 hours at room temperature to achieve equilibrium. Diluted digesta of each sample were dry-ashed to determine total phosphorus content after the *in vitro* digestion process. Briefly, 2-3g of diluted digesta were pipetted into porcelain crucibles and placed in a muffle furnace. Samples were heated to 60°C at a rate of 3°C per minute and held at 60°C for 24 hours, then ramped to 600°C at a rate of 3°C per minute and held at 600°C for 24 hours. Dry-ashed samples were dissolved in 1 mL of concentrated (70%) nitric acid and diluted to 25 mL with ultrapure water (final nitric acid concentration 2.8%). Dialysate and dry-ashed diluted digesta were analyzed for phosphorus content using MP-AES. Percent bioaccessibility was determined by calculating the fraction of dialyzable phosphorus from the digesta. Data is reported on an “as prepared” basis for all food products. The bioaccessible and total phosphorus content of each product is reported as mg per 100g, unless otherwise reported.

Statistics

Data were analyzed using Microsoft® Excel® (Version 2303). Descriptive statistics were used to characterize data among protein sources (soy, pulse, and animal) and within each food category (beef, dairy, sausage/bacon, chicken/turkey, natural and traditional forms). Regression analysis was performed to determine the relationship between percent and absolute

bioaccessible phosphorus for each food category. A p-value of <0.05 was considered statistically significant.

Results

Beef and Beef Alternatives

Average percent bioaccessible phosphorus was lowest in pulse-based (32%), followed by soy-based (52%) and highest in animal-based beef products (89%) (**Table A.C.S3**). However, the average absolute bioaccessible phosphorus was similar between pulse- and soy-based beef (83 mg/100g and 90 mg/100g) (**Figure 3.2A, Table A.C.S3**). Both of which were lower than the average absolute bioaccessible phosphorus from animal-based beef (147 mg/100g). There was a strong correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the beef and beef alternative products evaluated (R=0.90, p=0.0001) (**Figure 3.2B**)**.** In the beef and beef alternatives category, only one animal-based product was identified as having at least one inorganic phosphate-containing additive on the ingredient list. The percent bioaccessible phosphorus of this product was 79% compared with an average percent bioaccessibility of 46% (range 23-99%) in products without an inorganic phosphate-containing additive identified on the ingredient list.

Milk and Milk Alternatives

Average percent bioaccessible phosphorus was higher in pulse-based milk alternatives (103%) than animal (cow's) milk (85%) and soy-based milk products (53%) (**Table A.C.S3**). Average absolute bioaccessible phosphorus in

soy-based milk (52 mg/100g) was lower but comparable to cow's milk (63 mg/100g) (**Figure 3.2C, Table A.C.S3**). Notably, average absolute bioaccessible phosphorus of pulse-based milk alternative products was ~2.5x higher than soy-based milk and cow's milk (150 mg/100g) (**Figure 3.2C, Table A.C.S3**). There was a strong correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the milk and milk alternative products evaluated (R=0.93, p=0.002) (**Figure 3.2D**). In the milk and milk alternatives category, two soy-based and three pulse-based milk alternative products were identified as having at least one inorganic phosphate-containing additive on the ingredient list and had an average percent bioaccessible phosphorus of 83%. This is higher than the average percent bioaccessible phosphorus of products without inorganic phosphate additives identified (70%). But, notably, the average percent bioaccessible phosphorus of soy-based milk alternative products were similar with and without additives (52% and 55%, respectively).

Other Dairy and Dairy Alternatives

Average percent bioaccessible phosphorus was 63% in pulse-based cheese which was lower than animal-based cheese (89%). Additionally, average absolute bioaccessible phosphorus of pulse-based cheese products was ~2x lower than animal-based cheese (152 mg/100g and 359 mg/100g) (**Table A.C.S3**). Percent and absolute bioaccessible phosphorus were negligible for soy-based cheese as both products had minimal amounts of total phosphorus (~5-7 mg/100g) and protein. Percent bioaccessible phosphorus and absolute

bioaccessible phosphorus were comparable between the soy-based yogurt (74% and 93 mg/100g) and animal-based yogurt (82% and 86 mg/100g) (**Table A.C.S3**).

Sausage and Bacon and Sausage and Bacon Alternatives

Average percent bioaccessible phosphorus was lowest in pulse-based (57%), followed by soy-based (88%) and highest in animal-based sausage and bacon products (104%) (**Table A.C.S3**). Pulse-based products had the lowest average absolute bioaccessible phosphorus (123mg/100g), whereas absolute bioaccessible phosphorus for soy-based and animal-based sausage/bacon products were similar (172 mg/100g and 176 mg/100g, respectively) (**Figure 3.2E, Table A.C.S3**). There tended to be a modest correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the sausage and bacon products evaluated (R=0.56, p=0.06) (**Figure 3.2F**). Of the sausage and bacon and alternatives category, one soy-based, one pulse-based, and one animal-based product were identified as having at least one inorganic phosphate-containing additive on the ingredient list. The average percent bioaccessible phosphorus of products containing at least one inorganic additive was 90% compared with 77% in products without an inorganic phosphate-containing additive identified on the ingredient list.

Chicken and Turkey and Chicken and Turkey Alternatives

Average percent bioaccessible phosphorus was comparable for pulse-based and soy-based chicken/turkey products (78% and 80%) while animal-

based chicken/turkey had the highest average percent bioaccessible phosphorus (110%) (**Table A.C.S3**). Average absolute bioaccessible phosphorus was 106 mg/100g for pulse-based chicken/turkey, ~1.3x and 2.3x lower than soy-based and animal-based chicken/turkey (135 mg/100g and 235 mg/100g) (**Figure 3.2G, Table A.C.S3**). The correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the chicken and turkey products evaluated was not significant (R=0.56, p=0.15) (**Figure 3.2H**). In the chicken and turkey and alternatives category, one soy-based, two pulse-based, and one animal-based product were identified as having at least one inorganic phosphate-containing additive on the ingredient list. The average percent bioaccessible phosphorus of products containing at least one inorganic additive was 85% compared with 96% in products without an inorganic phosphate-containing additive identified on the ingredient list.

Natural Forms and Traditional Processed Plant-Based Protein Products

Average percent bioaccessible phosphorus was lower in natural forms and traditional soy products (i.e., tofu, tempeh, soybeans etc.) compared with pulse products (i.e., chickpeas, green lentils, fava beans etc.) (56% and 83%, respectively) (**Table A.C.S3**). Of the pulse products evaluated, the average percent bioaccessible phosphorus was considerably higher in canned products than in prepared dried pulse products (103% and 64%, respectively), but absolute bioaccessible phosphorus was similar (64 mg/100g and 86mg/100g) (**Table A.C.S3**). In contrast to average percent bioaccessible phosphorus,

average absolute bioaccessible phosphorus was lower in pulse compared with soy natural form/traditional products (87 mg/100g and 154 mg/100g) (**Figure 3.2I, Table A.C.S3**). The correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the traditional soy and pulse products evaluated was not significant (R=0.27 p=0.39) (**Figure 3.2J**). Average percent and absolute bioaccessible phosphorus were higher in soy butter (88% and 309 mg/100g) than its comparison product peanut butter (70% and 226 mg/100g) (**Table A.C.S3**). Soy-based and pulse-based pastas had lower percent bioaccessible phosphorus but higher absolute bioaccessible phosphorus (47% and 97 mg/100g for soy-based, and 77% and 77 mg/100g for pulse-based) compared to the comparison wheat-based pasta (95% and 62 mg/100g). (**Table A.C.S3**). None of the natural forms and traditional processed plant-based protein products had inorganic phosphate-containing additives on the ingredient lists.

Discussion

In the present study, we found that most of the plant-based protein alternative products evaluated had lower absolute phosphorus bioaccessibility (mg/100g) than animal-based protein products. Additionally, we found a wide range of phosphorus bioaccessibility in the plant-based protein products, from ~27% in pulse-based beef products to ~100% in pulse-based milk. This may be attributed to the degree of remaining phosphorus bound in phytic acid following processing, differences in formulation, food matrices, and processing techniques.[263] We also observed a stronger correlation between percent and

absolute bioaccessible phosphorus for beef and milk and their plant-protein alternative products than in other food categories. This is likely because all products evaluated in these two categories were variations of either ground beef or cow's milk whereas products in other categories spanned a larger variety of forms (i.e., chicken breast versus deli-meat). The strong correlation found in the beef and milk food categories indicates that a higher percent bioaccessible phosphorus would be associated with higher absolute bioaccessible phosphorus. However, in the food categories where non-significant correlations were observed, a higher percent bioaccesible phosphorus would not necessarily be predictive of higher absolute bioaccessible phosphorus. Taken together, these data highlight the need for further investigation of phosphorus bioaccessibility in plant-based protein products before recommendations can be made for patients with CKD as it is likely that some products may have desirable levels of phosphorus bioaccessibility while others may be undesirable.

Notably, pulse-based milk had ~2.5x more absolute bioaccessible phosphorus in comparison to cow's milk and soy-based milk alternatives. This was despite *both* soy-based and pulse-based milk products containing at least one inorganic phosphate additive. Interestingly, the soy-based milk products with phosphate additives contained tricalcium phosphate while all pulse-based milk products contained dipotassium phosphate. This provides evidence that not all inorganic phosphate-containing food additives contribute equally to phosphate burden, but rather that inorganic phosphate salts with varying solubility

presumably have differences in bioaccessibility. In contrast, soy-based milk and cow's milk had similar absolute bioaccessible phosphorus. However, when protein content from the Nutrition Facts Label is taken into account, soy-based milk in this study actually had a more desirable bioaccessible phosphorus-to-protein ratio of 6.9 mg/g compared to cow's milk with 18.6 mg/g. Therefore, soy-based milk may be a more optimal choice for patients with CKD on dialysis who are advised to consume high protein (1.0-1.2g/kg/d) but reduced phosphorus diet[121] (total phosphorus-to-protein ratio of ≤10-12 mg/g).[264,265]

The data presented in this study also demonstrates the differences in phosphorus bioaccessibility of cooked natural or traditional forms of plant protein foods (soy versus pulse). Percent bioaccessible phosphorus was lower in soy products than pulse products, but the absolute bioaccessible phosphorus was lower in pulse compared with soy products. While the average percent bioaccessible phosphorus for legumes in this pilot study was higher (56%, range 48-63% for soy and 83%, range 61-103% for pulses) than previously described by Karp et al.[216] (38%, range 6-42%), it is important to note that the products evaluated by Karp et al.[216] were uncooked or raw. In addition, we found that canned pulse-products, which did not list any inorganic phosphate additives on the label, had a higher average percent bioaccessible phosphorus but similar average absolute bioaccessible phosphorus to their prepared dried pulse-product comparators (103% versus 64% and 64 mg/100g versus 86 mg/100g). Therefore, our data demonstrate that, indeed, processing, soaking and cooking

of natural or traditional forms of plant-based protein can impact phosphorus bioaccessibility. One reason for this may be the effect that processing methods have on phytic acid content. This was demonstrated by Pal et al.[266] in which phytic acid was reduced by dehulling (-52% to -60%), germination (-40% to -59%), and cooking (-32% to -44%) in five varieties of lentils.

This is particularly relevant for emerging processed plant-based protein products which undergo a variety of processing techniques[267,268] to aid in optimal texture, structure and functionality, so that they closely mimic that of their animal-based counter products. The processing techniques used on these products may lead to the hydrolysis or reduction of phytic acid, thereby increasing accessible phosphorus.[209,269,270] Indeed, extrusion of plant proteins has been found to decrease phytic acid anywhere from 18-99%, depending on the processing conditions and material matrix.[271–273] Phytic acid may be further reduced by the consumer with cooking methods such as boiling or microwaving.[274]

Processing may also include the addition of inorganic phosphate additives[161,162,275], which are often assumed to have nearly 100% phosphorus bioaccessiblity.[276,277] However, our data indicate that the presence of inorganic phosphate additives does not necessarily lead to high phosphorus bioaccessibility of the product. In particular, the soy-based milk products with and without phosphate additives had comparable phosphorus bioaccessibility. The observation that inorganic phosphate is not 100% bioavailable has also been seen by others.[67,278] Parfitt et al.[278] found that phosphorus from sodium

phosphate was absorbed 75-89% in n=5 individuals. More recently, Scanni et al.[67] found that only 73% of phosphorus from sodium phosphate administered via the gastrointestinal tract was recovered in the urine. Thus, more research on how processing techniques and phosphate additives impact phosphorus bioaccessibility is warranted.

Understanding the relationship between total, percent bioaccessible, and absolute bioaccessible phosphorus and serving size is critical when recommending foods to patients with CKD. Although the percentage of bioaccessible phosphorus may be high, the total phosphorus may be low, contributing to an overall lower absolute bioaccessible phosphorus load. For instance, we found that the percent bioaccessible phosphorus of canned chickpeas was 102%, but the absolute bioaccessible phosphorus was only 53 mg/100g. An example of how this relationship translates to serving size can be found in **Table 3.1.** Total phosphorus per serving size was comparable between a cheese, pulse-based beef, and tofu product. However, absolute bioaccessible phosphorus of the pulse-based beef and tofu product were substantially lower (31 mg/serving size and 58 mg/serving size) compared with a cheese product (100 mg/serving size). Interestingly, total phosphorus per serving size for Cola was ~1.6 to 2 times lower than the cheese, pulse-based beef and tofu product. But, absolute bioaccessible phosphorus for Cola was similar to tofu (57 mg/serving and 58 mg/serving).

Although some emerging processed plant-based protein products may contain lower bioaccessible phosphorus, very little is known about how these alternative products impact health outcomes. In a randomized crossover trial of 36 healthy adult participants, it was observed that 8 week consumption of ≥ 2 servings of plant-based meat alternatives led to improvements in trimethylamine-N-oxide (TMAO), LDL-cholesterol and body weight.[279] In a secondary analysis of this same study, negligible improvements were observed for inflammatory markers.[280] Therefore, further investigation of the health benefits and potential harms of consuming plant-based protein alternative products is required.

Strengths and Limitations

One strength of the current study was that the *in vitro* digestion experimental protocol was based on the INFOGEST protocol.[262] This is an internationally developed *in vitro* digestion protocol that has been standardized to accurately simulate adult human gastrointestinal digestion. Thus, the data presented here are directly applicable to this population. Additionally, a wide variety of food categories (beef, dairy, sausage/bacon, and chicken/turkey) were evaluated. These products were chosen for determination of bioaccessible phosphorus based on retail sales data[250] and popular demand and are likely to be representative of foods consumed in American diets.

As a pilot investigation, a limitation was the small number of products evaluated per food category. Due to this and, in some cases, heterogeneity of samples within categories, we chose to do descriptive statistics only. Further

studies with a more comprehensive representation of samples within these categories would be needed to perform appropriate statistical analysis for differences between means. Another limitation was that we had only one sample of each product, so we were not able to determine within product variation. Product variation may exist for a variety of reasons including different product lots and/or manufacturing locations. Differences in soil phosphorus content could also impact the amount of phosphorus found in these plant-based proteins.[281,282] Another limitation of the present study was that a static rather than dynamic model was used to perform the *in vitro* digestion experiments. Dynamic models can better replicate the complexity of the gastrointestinal tract as they offer greater control of pH drift, simulation of gastric transit and peristalsis, and are able to simulate digestion in the large intestine.[283] In addition, static and dynamic methods performed *in vitro* may not reflect *in vivo* bioaccessibility as individual people have different efficiencies for making phosphorus accessible for absorption. However, data from this study of *in vitro* bioaccessibility provides valuable information on phosphorus bioaccessibility of a wide variety of plant-based protein products and their animal protein counterparts under standardized conditions that can be used to generate hypotheses to drive future work in this area.

Practical Application

Overall, most soy- and pulse-based protein products offered lower bioaccessible phosphorus per 100g serving than their animal counterparts. Thus,

blanket dietary recommendations to avoid processed plant-based protein products may be unnecessary and may eliminate would-be beneficial food choices This pilot investigation suggests that it may be suitable for healthcare professionals to recommend certain processed plant-based protein products for inclusion in the diets of patients with CKD to aid in the management of dietary phosphorus consumption. However, more data are needed to inform such dietary guidance.

Figure 3.1. Total and bioaccessible phosphorus determination schematic

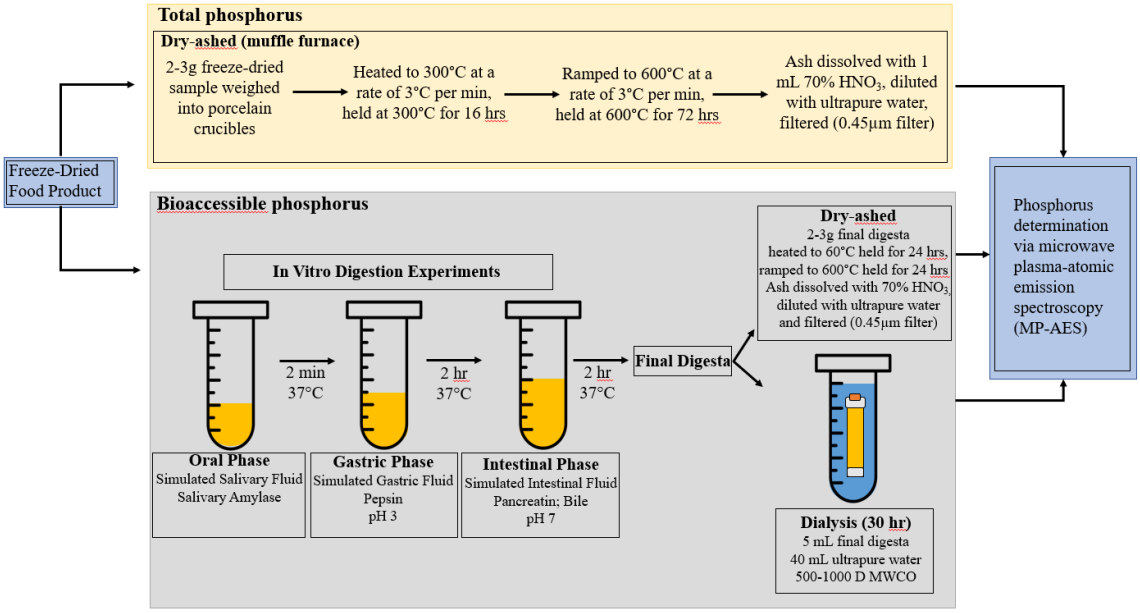
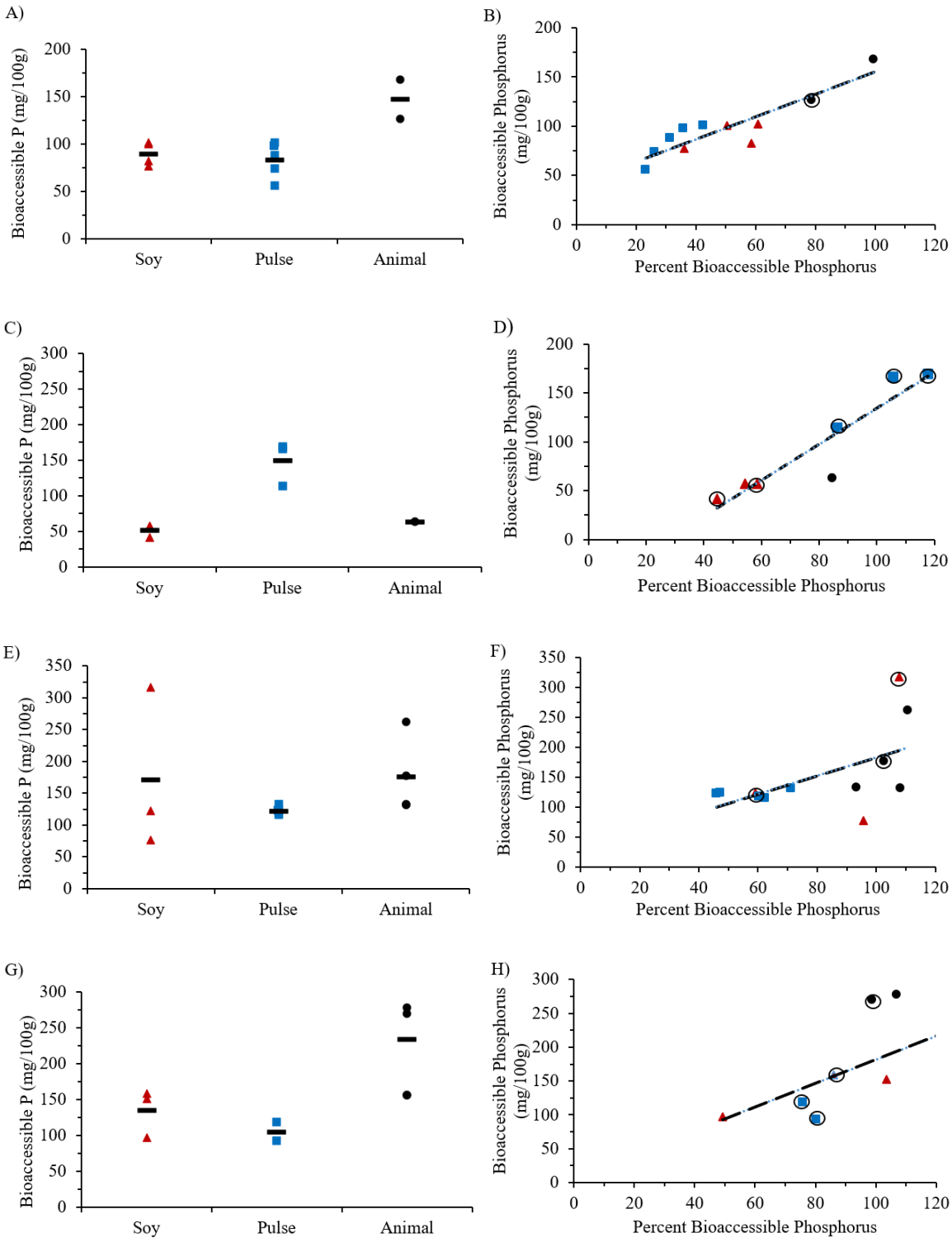


Figure 3.1. Total and bioaccessible phosphorus determination schematic

Forty-six total products including 17 pulse-based, 16 soy-based and 13 animal-based proteins along with 17 natural and traditional forms of plant protein products (e.g., tofu) were purchased from nationwide retailers, cooked according to package directions and freeze dried prior to total and bioaccessible phosphorus determination. Total phosphorus was determined from freeze-dried samples (pre-digestion). Bioaccessible phosphorus was determined from freeze-dried samples that were put through an *in vitro* digestion experiment to simulate human gastrointestinal digestion. Final digesta were subsequently dialyzed and both dialysate and final digesta were analyzed for phosphorus content.

Figure 3.2. Phosphorus bioaccessibility



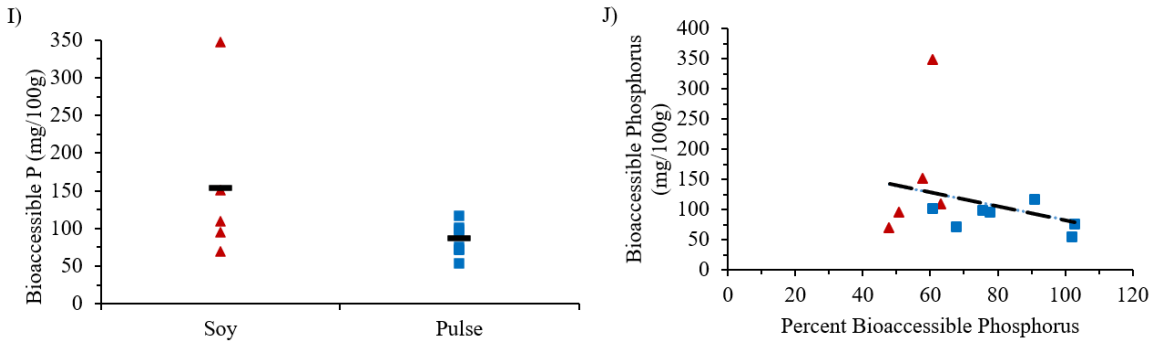


Figure 3.1. Phosphorus bioaccessibility.
A-B) Phosphorus bioaccessibility of beef and beef alternative products. Average absolute bioaccessible phosphorus was similar between soy and pulse-based beef. A strong correlation between percent and absolute bioaccessible phosphorus was observed for beef and beef alternative products. **C-D) Phosphorus bioaccessibility of milk and milk alternative products.** Soy-based milk alternative products and cow's milk had comparable average absolute bioaccessible phosphorus. A strong correlation between percent and absolute bioaccessible phosphorus was observed for milk and milk alternative products. **E-F) Phosphorus bioaccessibility of sausage/bacon and sausage/bacon alternatives.** Average absolute bioaccessible phosphorus was lower in pulse-based products compared with soy-based and animal products. There tended to be a modest correlation between percent and absolute bioaccessible phosphorus for sausage/bacon and sausage/bacon alternative. **G-H) Phosphorus bioaccessibility of chicken/turkey and chicken/turkey alternatives.** Average absolute bioaccessible phosphorus was lower in pulse-based followed by soy-based and highest in animal-based chicken and turkey products. The correlation between percent and absolute bioaccessible phosphorus for chicken/turkey and

chicken/turkey alternative products was not significant. **I-J) Phosphorus bioaccessibility of natural form and traditional plant-based proteins.** Average absolute bioaccessible phosphorus was lower in pulse natural form/traditional products than soy. The correlation between percent and absolute bioaccessible phosphorus for natural form and traditional plant-based products was not significant. Triangles represent soy-based products, squares represent pulse-based products, and circles represent animal-based products. Black circle outlines represent the presence of at least one inorganic phosphate additive on the ingredient list of the product. Average values for graphs A, C, E, G, and I are indicated by a vertical black line.

Table 3.1. Total and bioaccessible phosphorus by serving size

Product	Serving Size (g)	Total Phosphorus (mg/serving)	Percent Bioaccessible Phosphorus	Absolute Bioaccessible Phosphorus (mg/serving)
Cheese Shreds	28	113	89	100
Plant-Based Beef	55	133	23	31
Tofu	84	122	48	58
Cola[216]	355	67	84	57

[216] Data for Cola generated from Karp and colleagues.

Table 3.1. Total and bioaccessible phosphorus by serving size.

Total phosphorus per serving was similar between a cheese, plant-based beef, and tofu product. However, absolute bioaccessible phosphorus was substantially lower per serving in the plant-based beef and tofu compared with the cheese shreds.

Chapter 4: Effects of Plant-Based Protein Consumption on Kidney Function and Mineral Bone Disorder Outcomes in Adults with Stage 3-5 Chronic Kidney Disease: A Systematic Review

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Abstract

Introduction: Plant-based protein is of growing interest for dietary management of chronic kidney disease (CKD) and is hypothesized to preserve kidney function and reduce CKD-mineral bone disorder (MBD) complications among other benefits. This systematic review aimed to summarize the available clinical trial evidence for the effect of plant-based protein on kidney function and CKD-MBD outcomes in adults with stage 3-5 CKD not on dialysis.

Methods: Searches of Medline, Embase, Agricola, CAB abstracts, Web of Science, Scopus, and hand searching were performed. Clinical trials with ≥8 participants ≥18 years of age with an eGFR <60 mL/min/1.72m2 but not on dialysis were included. Additionally, only clinical trials with ≥1-week interventions with ≥50% dietary protein from plant-based sources and reported at least one outcome for both kidney function and CKD-MBD outcomes were included. Of

10,962 identified abstracts, 32 met inclusion criteria and were assessed for risk of bias.

Results: Results for kidney function and CKD-MBD outcomes were heterogenous, with most studies having suboptimal methodological quality. In most of the studies (27/32), protein source was altered only secondarily to low protein diet interventions. Thus, data synthesis and interpretation were focused on a subset of five studies that investigated a change in protein source only (i.e., animal vs plant). Of this subset, four studies reported no change in kidney function while one study reported a decrease. Three studies reported no change in serum phosphorus and one study reported lower serum phosphorus following a vegetarian diet. Further, limited data and inconclusive results were observed for phosphaturic hormones, PTH and FGF-23.

Conclusion: Current clinical trial evidence on plant-based protein interventions for preserving kidney function and preventing CKD-MBD is limited to inform clinical guidelines at this time. This systematic review emphasizes the on-going need to research the effects of plant-based protein on kidney function and CKD-MBD outcomes.

Introduction

The global prevalence of chronic kidney disease (CKD) is ~697.5 million people, including ~37 million adults in the United States.[7,284] As kidney function declines, disturbances in calcium (Ca) and phosphorus (P) metabolism result in CKD-mineral bone disorder (CKD-MBD).[285,286] CKD-MBD is characterized by mineral metabolism abnormalities, including increased fibroblast growth factor-23 (FGF-23), decreased 1,25-dihydroxyvitamin D (1,25D), increased parathyroid hormone (PTH), and, eventually, hyperphosphatemia and hypocalcemia.[113,287,288] These abnormalities are associated with an increased risk of adverse cardiovascular events, vascular calcification, bone abnormalities, fragility fractures, and mortality.[14,18,107,118,289–293]

Nutrition therapy is a cornerstone of disease management in CKD and in the prevention of CKD-MBD progression. The 2020 update of the Kidney Disease Outcomes Quality Initiative (KDOQI) Clinical Practice Guideline for Nutrition in CKD recommends a protein intake of 0.55-0.60 g/kg/d for stage 3-5 CKD (not on dialysis, and without diabetes).[121] This recommendation was given the highest grade of evidence (1A). A low protein diet (LPD) preserves kidney function by decreasing intraglomerular pressure as well as improving uremia and metabolic acidosis.[123] A recent systematic review and meta-analysis showed that a LPD (<0.8 g/kg/d) led to a significant reduction in progression to end-stage kidney disease (ESKD) compared with higher protein consumption (>0.8

g/kg/d).[124] Further, a very low protein diet (VLPD) (<0.4 g/kg/d), showed even greater preservation of kidney function compared with a LPD.[124]

Less is understood about the influence of protein source (i.e., plant vs animal) on disease outcomes in people with CKD. The previous KDOQI guidelines from 2000 recommended that people with pre-dialysis CKD consume at least 50% of their dietary protein from high biologic value sources (i.e., animal-based proteins) but this was opinion-based and supporting evidence was not evaluated.[294] However, the new 2020 KDOQI guidelines evaluated evidence for the question, “What is the effect of protein type (animal vs plant) intake on outcomes in adults with CKD 1-5D, nondialysis and transplant?” This resulted in the change from the 2000 guidelines as it was found that there is insufficient evidence available to recommend a particular protein source (plant vs animal).[121]

More broadly, plant-based diets are growing in popularity among consumers,[267] and enthusiasm for these diets are growing among kidney professionals based on the potential health benefits in people with CKD.[195,196,251,295,296] Plant-based diets are usually lower in saturated fat and dietary cholesterol, higher in mono- and poly-unsaturated fat and dietary fiber; have anti-inflammatory properties; and have the potential to reduce metabolic acidosis in individuals with CKD.[121,195,199,251] Additionally, differences in bioavailable phosphorus in plant-based protein compared with animal-based protein is likely a major factor responsible for possible effects of these diets on CKD-MBD outcomes. It is generally assumed that phosphorus bioavailability is

lowest from plant sources due to high phytic acid content, followed by organic bound phosphorus from animal sources, and highest from inorganic phosphate-containing food additives.[22,135,297] However, estimates of percent bioavailability vary greatly, and most data available are on phosphorus bioaccessibility[216,217] (the phosphorus accessible for absorption) rather than true bioavailability (the phosphorus that is absorbed and available for use by the body).[253]

Observational studies have supported health benefits of plant-based protein intake. A cross-sectional study in which participants were stratified by estimated glomerular filtration rate (eGFR), showed that with every 33% increase in the ratio of plant protein to total protein, there was a 19% lower mortality risk in those with eGFR <60 mL/min/1.73m^2. [298] In a different study of 5,316 adults, each 20 g increase in plant protein intake was associated with a 16% decrease in CKD incidence, suggesting that plant-based protein consumption may play a role in kidney function preservation.[299] Additionally, a cross-sectional study showed that those who consumed a lacto-ovo vegetarian diet had significantly lower serum phosphorus compared with those who consumed an omnivorous diet.[300] Further, from the Chronic Renal Insufficiency Cohort study, higher percent plant-based protein consumption was associated with lower serum FGF-23 but not serum phosphorus or PTH.[301]

These observational studies suggest a possible benefit of consuming more plant-based protein compared with animal-based protein for preservation of kidney function and reduced signs of CKD-MBD. The new 2020 KDOQI guideline

reported limited evidence for the effects of protein source on traditional biochemical markers of nutrition status and inflammation, lipid panel measures, and serum calcium and phosphorus in CKD 1-5D and posttransplantation. Five randomized controlled trials published between 1998-2016 met their search and screening criteria and were evaluated in developing the guideline.[121] However, outcomes of kidney function (e.g., creatinine clearance, eGFR, BUN, serum creatinine) and CKD-MBD (e.g., PTH, FGF-23, 1,25D, bone or cardiovascular outcomes) were not addressed. Thus, this systematic review aimed to evaluate the clinical trial evidence on the effect of dietary plant-based protein on kidney function and CKD-MBD outcomes in adults with stage 3-5 CKD.

Key Question:

1. What is the effect of dietary plant-based protein on kidney function and CKD-MBD outcomes in adults with stage 3-5 CKD (**Figure 4.1**)?

Methods

Search Strategy

Using the Ovid interface, the following databases were searched: Medline (1966-July 2021), Embase (1947-July 2021), Agricola (1970-July 2021), CAB Abstracts (1973-July 2021), Web of Science (1900-July 2021) and Scopus (1966-July 2021). These databases were searched for all studies investigating CKD and the incorporation of plant-based protein sources. MeSH terms and keywords were incorporated based on the specifications of each database. Other searches conducted included hand searching of ClinicalTrials.gov and reference lists from included studies and related systematic reviews. Search strategies that

were used were reviewed by a reference librarian at the University of Minnesota (**Table A.D.S1**).

Study Selection Process

Three reviewers conducted title and abstract and full-text screening for inclusion. All screening processes took place using the PICO Portal platform.[302] Eligible study designs included randomized and unrandomized, blinded and unblinded clinical trials as well as cross-over trials. Studies were included if they investigated at least 8 participants with stage 3-5 CKD not on dialysis and were published in a peer-reviewed journal in English. Studies were excluded if they were conference abstracts or letters to the editors. Disagreements in study selection were discussed between the three reviewers until resolution was met.

Participant Characteristics

Trials considered for inclusion included participants ≥ 18 years of age with stage 3-5 CKD who were not on dialysis. The stages of GFR were defined according to the 2012 Kidney Disease Improving Global Outcomes Clinical Practice Guideline for the Evaluation and Management of CKD where stage 3 is 30-59 mL/min/1.73m2, stage 4 is 15-29 mL/min/1.73m2, and stage 5 is <15 mL/min/1.73m^2.[8]

Hemo- and peritoneal dialysis patients along with kidney transplant recipients were excluded as they represent a subpopulation with different nutritional needs and potential complications that are outside the scope of this systematic review.

Intervention Characteristics

Studies that incorporated at least 50% of protein from plant-based sources for ≥ 1 week were considered for inclusion. Trials were not excluded based on comparator nor setting of the trial.

Outcomes

As this systematic review is intended to deepen the understanding of available outcomes in the literature, studies were not excluded on the presence or absence of any outcomes. However, studies were required to have at least one outcome from both categories: kidney function or damage (e.g., creatinine clearance (CrCl) or eGFR, plasma/serum creatinine plasma/serum BUN or urea, proteinuria) and CKD-MBD (e.g., plasma/serum phosphorus, PTH, Ca, urine phosphorus) outcomes for assessment. A full list of outcomes included in each category are found in Supplementary Files 3 and 4.

Data Extraction

Relevant data from included literature were extracted independently by two reviewers. Contradictions in data extraction were resolved by a third reviewer when necessary. Data extracted included:

1. Publication year, study design, length of intervention, sample size
2. Participant characteristics (Ex. kidney function estimate, age, sex (M/F), race, diabetes prevalence)
3. Intervention/comparison, prescribed supplements, compliance
4. Outcomes of interest: kidney function and CKD-MBD related outcomes

Risk of Bias Assessment

Three reviewers independently assessed the quality of methodological approaches used by each study. Risk of bias was assessed using the ROB-2 tool for randomized controlled trials (RCT) and crossover trials.[303] All criteria were weighed equally in determining the overall risk of bias for each study. High risk of bias was determined when a study failed to meet a majority of the criteria or if criteria were unable to be clearly determined. Moderate risk of bias was determined when a study met the majority of the criteria and with no major determinable flaws or oversight. Low risk of bias was determined if a study met all criteria. Disagreements for risk of bias assessment were discussed between the three reviewers until resolution was met.

Data Synthesis Strategy

Analysis was limited to qualitative synthesis of the evidence as the heterogeneity of included studies was not appropriate for meta-analysis. Synthesis was focused on determining outcomes that have been studied in this area, the observed direction of change for reported outcomes, and the consistency of these findings across studies. When appropriate, effect size was estimated by Hedges' g calculations for studies that reported means and standard deviations for outcomes. Estimated effects were reported as small (g=0.2-0.49), medium (g=0.5-0.79) or large (g ≥0.8).[304] Studies were divided into 3 categories according to duration of intervention including short term (1-4 weeks), mid-length (>4-26 weeks), and long term (>26 weeks) to yield more comparable results. Values for outcomes were converted to U.S. conventional

units when necessary. If no known value was reported for an outcome, values were estimated from figures when available. Presented data is shown as mean ± SD unless otherwise stated. The preregistered protocol for this systematic review can be found on Open Science Framework Registries (DOI:10.17605/OSF.IO/GW2UB). Supplementary files 2, 3, and 4 can be found on Open Science Framework (https://www.doi.org/10.17605/OSF.IO/YFR57)

Strength of the Body of Evidence

Given the potentially confounding of interventions assessing both a change in protein amount and protein source, limited available evidence, and heterogeneity of included studies, a formal grading of the evidence was not feasible.

Results:

Literature Search Results

Through database and hand searching, a total of 10,962 records were identified for screening. Of these, 5,534 were duplicates and 5,428 underwent title and abstract screening. Upon review, 5,317 records were excluded at title and abstract screening and 111 remained for full text review. Seventy-nine studies were excluded during full text review for the following reasons: population (n=18), intervention (n=26), outcome (n=13), and study type (n=22). References for excluded studies can be found in **Appendix D.** Thus, 32 studies were included in this systematic review (**Figure 4.2**).[305–336]

Study Characteristics

Study characteristics can be found in **Table 4.1** and an expanded version can be found in **Supplementary File 2**. Study designs included, 1 case control trial,[319] 4 crossover trials,[306,307,309,313] 17 single arm (with one publication[326] reporting two single-arm trials),[305,312,314,316,318,322,324,325,327–333,335] and 10 parallel arm.[308,310,311,315,317,320,321,323,334,336] Of these, 9 were RCTs (the 4 crossover trials and 5 of the parallel arm studies).[306,307,309,311,313,319–321,334] Study locations included Brazil,[311] France,[308,314,317,322,324,325,327–329,331–333] Israel,[313] Italy,[306,309,310,312,315,316,318,319,323,326,330,334–336] Romania,[320,321] and the United States.[305,307] Study duration ranged from 1 week to 65 months. Three studies were categorized as short term (1-4 weeks),[305–307] 11 as mid-length (>4-26 weeks),[308–318] and 18 as long term (>26 weeks).[319–336] Seven studies included participants with diabetes mellitus,[305,307–309,330,335,336] a known major risk factor for the development of CKD. Notably, sample size varied drastically from N=8 to 159. Interventions varied in amount of protein provided, ranging from 0.3 g/kg/d plus ketoanalogue supplementation up to 0.9 g/kg/d. Importantly, most studies (27 of 32) investigated the effect of a change in protein amount (typically to a VLPD (~0.3-0.4 g/kg/d) in tandem with a change in protein source (i.e., from animal to plant). Thus, it was not possible in these studies to determine whether the observed effects on kidney function and CKD-MBD outcomes were due to changes in protein amount or protein source. However, there was a subset of five studies where the total protein amount was kept the same, allowing for a direct

comparison between animal and plant-based protein sources.[305,307,308,313,326] However, one of these studies[308] also supplemented the plant-based protein diet with ketoanalogue (KA) amino acids. These five studies will be the focus of this review as they address the key question without the confounding of protein amount. However, brief results will also be presented from the total 32 identified studies with more detailed information in the supplementary tables and files.

Risk of Bias

Twenty-eight studies were assessed by the RoB-2 tool for RCTs. Of these, 15/28 (54%) were considered moderate risk of bias[305,310,311,314,319–325,327,329,331,332] and 13/28 (46%) were considered high risk of bias.[308,312,315–318,326,328,330,333–336] This was generally due to concerns with randomization process (25/28 or 89%), deviations from intended interventions (20/28 or 72%), and selection of the reported results (20/28 or 72%). Missing outcome data was the lowest risk category with 23/28 (82%) receiving low concern for this criterion. (**Figure 4.3A, Figure A.D.S1A**).

The four crossover trials were assessed by the RoB-2 tool for crossover trials. One study was considered low risk of bias,[307] two were considered moderate risk,[306,309] and one was considered high risk of bias.[313] Missing outcome data and measurement of the outcome were the lowest concern categories (**Figure 4.3B, Figure A.D.S1B**).

For the subset of five studies where change in protein source from animal to plant was the main intervention, three were considered high risk of

bias,[308,313,326] one was considered moderate risk,[305] and one was considered low risk of bias.[307]

Kidney Function Outcomes

Kidney function outcomes qualitatively synthesized in this systematic review were CrCl or eGFR, serum creatinine, serum blood urea nitrogen (BUN) or urea, and proteinuria (kidney damage). Other outcomes reported for kidney function included serum protein, and urinary urea and urea nitrogen. A complete list of all reported kidney function outcomes can be found in **Supplementary File 3**. CrCl or eGFR and serum BUN or urea were the most widely reported outcomes across all studies with 27 of 32 reporting CrCl or eGFR and 24 of 32 reporting BUN or urea. However, results could not be pooled due to the heterogeneity of studies. A detailed description of the results for all 32 studies can be found in **Appendix D, Table A.D.S2**.

Of the subset of five studies where change in protein source from animal to plant was the main intervention, four studies[305,307,308,313] found no change in CrCl or eGFR (**Table 4.2, Supplementary File 3**). One long term study[326] reported a significant decrease in CrCl when subjects shifted from a mixed source low protein diet to a low protein vegetarian diet; however, this study had a small sample size (n=11) and lacked a control arm which contributed to its high risk of bias determination. A short duration study[305] found a small decrease in serum creatinine from baseline to endpoint following 4-weeks of a 70% plant-based protein diet, but this did not translate to a significant change in eGFR or

CrCl (**Table 4.2, Supplementary File 3**). One mid-length study[313] found significantly lower serum BUN with a large magnitude of effect after 6 months of a plant-based protein diet compared with a mixed protein diet, but this was also not associated with changes in kidney function (**Table 4.2, Supplementary File 3**). No changes in proteinuria were observed in the two studies[308,313] from the subset of five that reported on this outcome.

CKD-MBD Outcomes

CKD-MBD outcomes qualitatively synthesized in this systematic review were serum phosphorus, PTH, calcium, and urinary phosphorus excretion. Other outcomes reported for CKD-MBD include Ca x P product, serum FGF23, calcitriol, calcidiol, and alkaline phosphatase. A complete list of reported CKD-MBD related outcomes can be found in **Supplementary File 4**. Serum phosphorus and PTH were the most widely reported CKD-MBD related outcomes across all studies with 22 of 32 reporting serum phosphorus and 26 of 32 reporting serum PTH. Similar to kidney function outcomes, results for CKD-MBD outcomes could not be pooled due to the heterogeneity of studies. A detailed description of these results can be found in **Appendix D, Table A.D.S3**.

Of the subset of five studies where change in protein source from animal to plant was the main intervention, three studies[305,313,326] found no change in serum phosphorus (**Table 4.3**). One short term crossover study[307] of low risk of bias observed significantly lower serum phosphorus and FGF-23 following the vegetarian diet compared with the meat-based diet (**Table 4.3, Supplementary**

File 4). However, PTH was significantly higher with the vegetarian diet compared with the meat-based diet after one week, where PTH decreased over the week of intervention with the meat-based diet but did not change with the vegetarian diet. It should be noted that both the vegetarian and meat-based diets in this study were designed as low phosphorus diets (800 mg/d). In another short duration study,[305] of moderate risk of bias and with no control group, no significant change in PTH or iFGF23 from baseline to endpoint following consumption of a 70% plant-based protein diet was observed (**Table 4.3**). Fois et al.,[308] a mid-length study also observed no change in PTH with a plant-based protein diet. For 24-hour urinary phosphorus, two studies, one of short[305] and one of mid-length duration[313] observed a decrease with a large effect size following plant-based protein diet interventions and another short duration study[307] found no change (**Table 4.3**). For serum calcium, when reported, no change was observed with plant protein-based diet interventions (**Table 4.3**).[305,307,313]

Discussion

Overall, evidence from all 32 included studies is of suboptimal methodological quality and yields inconsistent results to provide firm conclusions on the effects of plant-based protein consumption on the kidney function and CKD-MBD outcomes assessed. Importantly, 27 of the 32 included studies were designed to investigate the effects of VLPD with ketoanalogue (KA) supplementation, and protein source was only altered as a byproduct of these interventions. Thus, the independent effect of plant-based protein source cannot

be distinguished from the effect of lower protein amount. Further, KA supplementation is most commonly in the form of calcium salts, providing up to ~600-1200 mg/d calcium[337,338] which could also affect mineral metabolism and confound the results of these studies. Also, several studies included vitamin D supplementation which could affect results as well. Thus, there is an on-going need to investigate the independent effects of plant-based protein on kidney function and CKD-MBD outcomes. Here we focus our discussion on the subset of five studies where change in protein source from animal to plant was the main intervention while protein amount is kept the same.

Of the subset of five studies where a change in protein source between animal to plant-based protein was the main intervention, short or mid-length duration studies[305,307,308,313] showed no change in kidney function (CrCl or eGFR), which is not surprising given that a longer period of time is likely needed to observe an effect on this outcome. Indeed, the long-term study by Barsotti et al.[326] was the only study to report a change in kidney function with a vegetarian low protein diet, but the change observed was a worsening, rather than improvement, of kidney function. However, with no parallel control arm, the decline in kidney function cannot be directly attributed to the intervention when the more likely explanation is a natural decline in kidney function over the duration of the >1 year study. In a pooled analysis of 8 crossover trials, Eckert et al.[339] found weak evidence for improvements in eGFR and urinary albumin excretion with a plant-based or white meat intervention in substitution of animal

or red meat in adults with diabetic kidney disease. Therefore, greater investigation of the effects of protein source on kidney markers in adults with stage 3-5 CKD is warranted.

In the present systematic review, we found that few studies reported on CKD-MBD outcomes aside from serum phosphorus and PTH. Of the subset of five studies, only one study found an effect of lower serum phosphorus with the plant protein intervention compared with animal protein. This study by Moe et al.[307] was the shortest duration of any study included, with diet interventions of only one week long. Yet, its strength of a randomized crossover design with controlled feeding allows for precise, known and consistent delivery of the intended intervention to evaluate efficacy. The other four studies[305,308,313,326] had longer durations (1 month to >1 year) but had less control of diet interventions, thus evaluating more effectiveness rather than efficacy. None of these longer studies found effects of the plant protein intervention on serum phosphorus. This suggests that a lower phosphorus (~800 mg/d) vegetarian diet may be efficacious in the short term for lowering serum phosphorus, but longer-term effectiveness of such a diet is not yet supported by currently available intervention data. This aligns with a conclusion from the 2020 KDOQI guidelines where a pooled analysis of results from Soroka et al.[313] and Moe et al.[307] (two studies also included in this systematic review) found no effect of the plant-based protein intervention on serum phosphorus.[121] Beyond the limited amount of evidence from intervention studies, a recent critical review[221] of intervention and

observational studies also concluded that evidence was mixed for a benefit of plant-based protein on serum phosphorus. However, detecting effects of dietary interventions on serum phosphorus in moderate CKD may be limited because serum levels are typically not yet elevated above the normal range.[107]

For serum PTH, Moe et al.[307] observed lower values after one week on the meat-based diet versus the vegetarian diet. Although this seems counterintuitive, both diets were low dietary phosphorus interventions, which would be expected to decrease serum PTH, regardless of protein source. However, the higher phytate content of the vegetarian diet likely reduced calcium bioavailability which could be expected to counteract the effect of low dietary phosphorus on serum PTH levels. This may have also contributed to the lack of effect of plant-based protein interventions on serum PTH in studies by Moorthi et al.[305] and Fois et al.[308] Additionally, the study by Fois et al. supplemented the plant-based diet with KA, providing 50 mg/d additional Ca per 8-10 kg body weight (~350 mg/d Ca for a 70 kg adult) which confounds effects of the plant-based diet on serum PTH, and no other mineral metabolism outcomes were reported. Baseline serum PTH is another factor which may affect results of these interventions, as levels were normal to modestly elevated in these studies of subjects with moderate pre-dialysis CKD.

Applicability

Results are applicable to adults with stage 3-5 CKD not on dialysis as studies with participants on hemo- or peritoneal dialysis were excluded.

Interestingly, only 7 studies included participants with diabetes mellitus. Thus, more research in this area is needed to broaden applicability of results to participants with comorbidities and CKD etiologies. Studies included were conducted in Brazil, France, Israel, Italy, Romania, and the United States and results are likely broadly applicable to these areas.

Strengths and Limitations

One strength of the current systematic review is the extensive search strategy utilized to yield all relevant literature in this topic area. This search strategy also provides a broader understanding of how plant-based protein consumption and CKD have been previously investigated. An additional strength is that studies were categorized by duration. This allows for comparison of results by duration category (e.g., short, mid-length, and long) and allows for comparison of results over time. One limitation of the current systematic review is that we were unable to assess potential publication bias, as some studies could have been missed during the literature search process. However, to the best of our knowledge, and with the help from a reference librarian, the search strategies utilized were tailored to yield all relevant literature in this topic area. Any missed literature would likely be publication of a small study and unlikely to shift the review findings. Our systematic review is also limited to the focus on effects of plant-based protein specifically on kidney function and CKD-MBD outcomes. Plant-based protein and/or plant-based diets may provide other health benefits in patients with CKD, such as improvements in metabolic acidosis, uremic toxins,

blood lipids, blood pressure, and inflammation,[251] but these outcomes were outside the scope of this systematic review.

Gaps in the Literature and Future Research Considerations

A limitation of the existing literature on this topic is that most studies altered protein source only in the context of low protein diet interventions. Therefore, we were unable to determine which dietary alteration (i.e., protein amount or protein source) contributed to changes in the kidney function and CKD-MBD outcomes assessed in 27 of the 32 included studies. Additionally, plant-based protein is only one component of plant-based eating (or plant-based diets), which can also be characterized by greater fruit and vegetable intake. But, there is no standard definition for a plant-based diet or optimal level of plant-based protein intake, which has been previously acknowledged by others.[251] This adds to the heterogeneity of available studies and the associated challenges with synthesizing the evidence in this area.

In addition, some studies may have only included participants who were highly motivated to comply with the intervention, which limits the generalizability of the findings. For example, Chauveau et al.[322] note that their study consisted of "only carefully selected, motivated, and regularly monitored patients." Lack of control groups and/or randomization in several of the available studies is another limitation. Moreover, ~63% (20/32) studies were conducted over 2 decades ago. Another limitation of the existing literature on this topic is the lack of CKD-MBD outcomes investigated. While most studies reported serum phosphorus and PTH,

other CKD-MBD outcomes were not consistently reported, including other relevant biochemistries (e.g., FGF-23 and 1,25D) and clinical outcomes for bone and cardiovascular disease.

Future research in this area should focus on interventions that are of longer duration, adequately powered and utilize randomization and allocation concealment to reduce risk of bias. These studies are needed to understand the impacts of plant-based protein compared with animal-based protein consumption on clinical outcomes (e.g., RRT initiation, cardiovascular events, fracture incidence, and mortality), nutritional adequacy, and potential harms such as hyperkalemia. This should be evaluated together with high quality observational research to strengthen the evidence base for drawing conclusions regarding the use of plant-based protein in CKD. Future research in this area should also strive to consistently report serum phosphorus, PTH, and other relevant CKD-MBD biochemistries that are lacking in the literature such as FGF-23 and 1,25D, as these are key intermediate outcomes in the development of CKD-MBD complications.

Practical Application

While there is promising observational evidence as well as biological plausibility for beneficial effects of plant-based protein for the dietary management of CKD, there is limited intervention study evidence for the effects of plant-based protein on kidney function and CKD-MBD outcomes at this time. In accordance with the current KDOQI guidelines, it is prudent to consider patient

preferences and individual risk factors when recommending increased plant-based protein in the dietary management of moderate CKD.

Figure 4.1. PICOTS framework analytical logic model

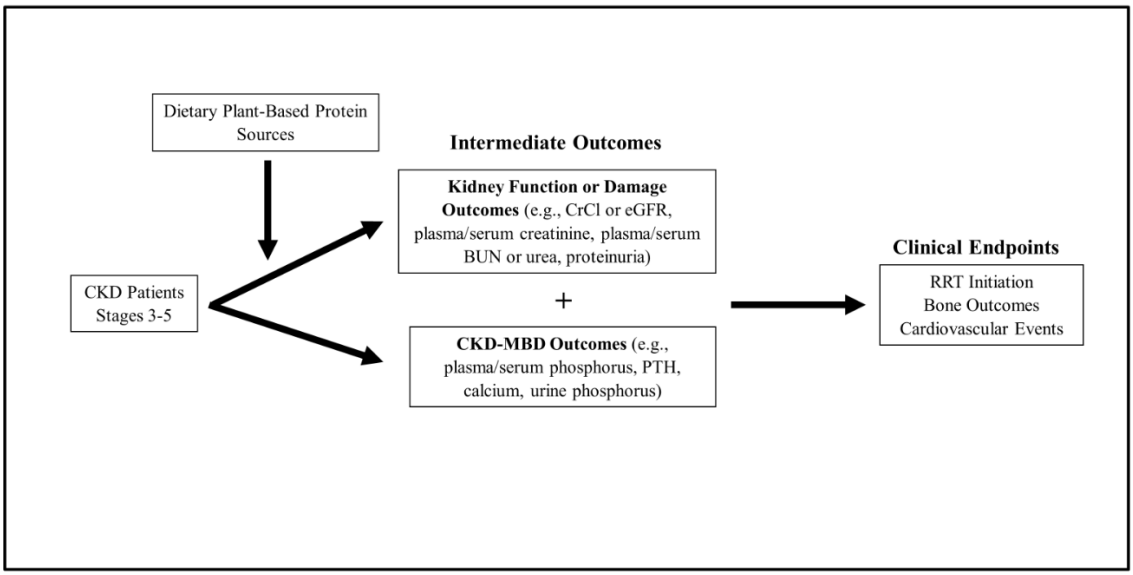

Figure 4.1. PICOTS framework analytical logic model
The key question of this systematic review is aimed at determining the effect of dietary plant-based proteins on kidney function and CKD-MBD outcomes. Examples from each category are shown here with a full list of outcomes provided in Supplementary file 3 and 4.

Figure 4.2. Flow chart of literature search and screening process

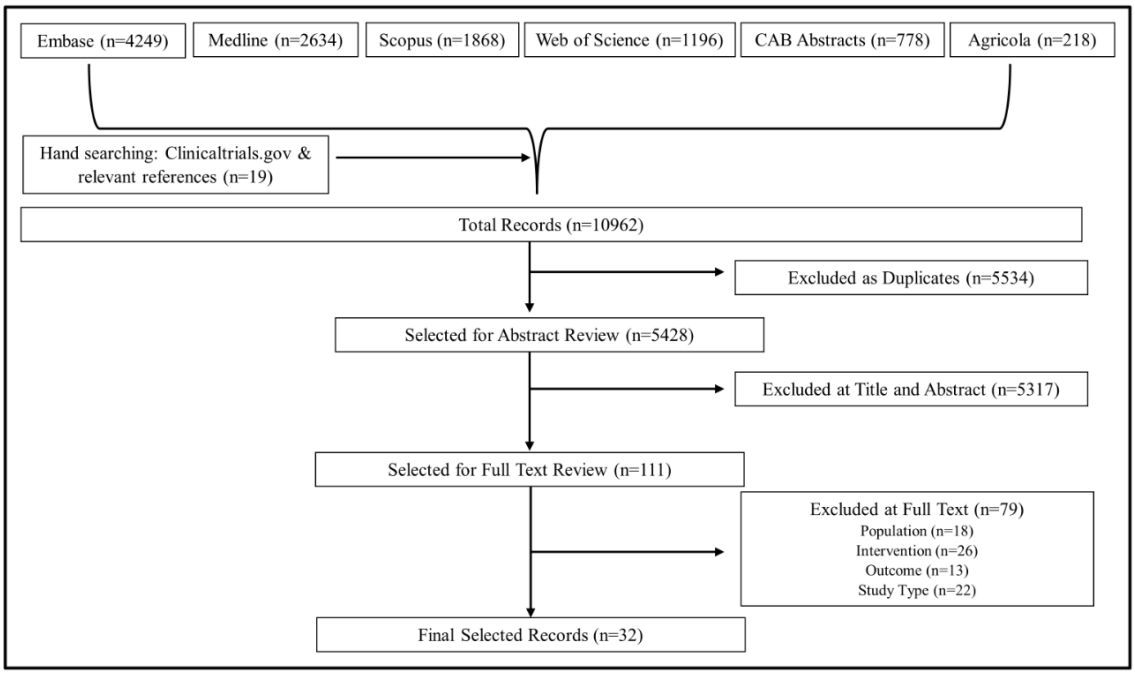

Figure 4.2. Flow chart of literature search and screening process

Table 4.1. Summary of study characteristics

Reference	Study Characteristics				Subject Characteristics					Diet Treatment					
	Study Duration Category	Length	*n*	Design	Kidney Func	Age (y)	Sex (M/F)	Diabetes	Race	Diet	Energy (kcal/kg/d)	Protein (g/kg/d)	Protein Source	Protein Suppl	Phosphorus (mg/d)
Moorthi 2014[305]	**Short term (1-4 weeks)**	4 wk	13	Single Arm	CrCl 51±17	54.8±13	6/7	4	7 C, 6AA	Veg.	NR	0.8-0.9	70% veg.	N	800-1300
Di Iorio 2012[306]		1 wk	32	RCT Crossover	CrCl 30±8	66±9	21/11	excl	NR	VLPD	≥ 30	0.3	66% veg.	Y	350-420
										LPD	≥ 30	0.6	52% animal	N	600-700
Moe 2011[307]		1 wk	8	RCT Crossover	eGFR 32.3±6	61±8	4/4	4	7 C, 1 H	Veg.	2094[d]	78.9[g]	95% veg.	N	813
										Meat	2181[d]	78[g]	79% animal	N	817
Fois 2019[308]	**Mid-length (>4-26 weeks)**	≥ 3 mo	65	Parallel Arm	eGFR 22 (7-57)	24-101[a]	82/49[a]	66[a]	NR	LPD, Veg.	30-35	0.6	veg.	Y	NR
										LPD, Mixed	30-35	0.6	50% animal	N	
										FD	30-35	0.8	NR	N	
Di Iorio 2018[309]		6 mo	60	RCT, Crossover	eGFR 15-45	66±16	46/14	24	NR	VLPD	30-35	0.3-0.5	veg.	Y	453±171
										MD	30-35	0.7-0.8	mixed	N	772±231
										FD	30-35	1	mixed	N	971±242

Bellizzi 2007[310]		6 mo	110	Parallel Arm	CrCl 17.1±5.5	58 ±16.1	18/12	NR	NR	VLPD	≥ 30	0.35	66 % veg.	Y	NR
					CrCl 18.2±6.0	56.3± 15.7	29/28			LPD	≥ 30	0.6	52 % animal	N	
					CrCl 17.6±5.3	56.3± 15.6	14/9			FD	≥ 30	UC	UC	N	
Feiten 2005[311]		4 mo	24	RCT, Parallel Arm	CrCl 16.7±5.3	49.7± 11.3	15/9	excl	NR	VLPD	30-35	0.3	veg.	Y	373 ±125
					CrCl 17.8±2.9	43.9± 16.3				LPD	30-35	0.6	NR	N	527 ±172
Barsotti 1998[312]		4 ± 2 mo	21	Single Arm	CrCl 7.2±3.3	56 ±13	12/9	NR	NR	VLPD	30-35	0.3	veg.	Y	5-6[e]
Soroka 1998[313]		6 mo	9	RCT, Crossover	CrCl 30.5±3.6	30-85	5/4	NR	NR	Veg.	32	0.75	soya	N	11[e]
										Mixed	32	0.75	50 % animal	N	
Rigalleau 1997[314]		3 mo	8	Single Arm	GFR 13.2±7.9	41.8± 16.4	7/1	excl	NR	VLPD	35	0.3	veg.	Y	5-7[e]
Bergesio 1995[315]		≥ 3 mo	21	Parallel Arm	SCr 8.4±2.2	56 ±9	9/4	excl	NR	VLPD	30-35	0.3	veg.	Y	NR
					SCr 7.8±1.7	63 ±7	4/4			LPD	30-35	0.6	NR	N	
Ciardella 1990[316]		4-8 mo	13	Single Arm	CrCl 13.4±2	18-41	13/0	NR	NR	VLPD	NR	0.3	veg.	Y	NR
Forget 1990[317]		6 mo	32	Parallel Arm	eGFR 16.2±4.6	54 ±7.7	NR	excl	NR	VLPD	35	0.4	95 % veg.	Y	NR
					eGFR 15.8±4.9	49 ±8.2				LPD	35	0.7	mixed	N	
Ciardella 1989[318]		1-4 mo	12	Single Arm	CrCl 7.2±2.4	18-42	12/0	NR	NR	VLPD	≥35	0.3	veg.	Y	350

Di Iorio 2017[319]	**Long term (> 26 weeks)**	12 mo	146	RCT, Case Control	CrCl 26±12	73.6±11.2	85/61	excl	NR	VLPD	2110±225[d]	0.3-0.4	90% veg.	Y	324±122
					CrCl 39±14					UPD	2320±207[d]	0.6-1.0	75% animal	N	335±141
Garneata 2016[320]		15 mo	159	RCT, Parallel Arm	eGFR 18.0 {15.5,20.1}[c]	55.2[f]	63% M	excl	W	VLPD	30	0.3	veg.	N	NR
					eGFR 17.9 {14.3,19.3}[c]	53.6[f]	59% M			LPD	30	0.6	NR	N	
Mircescu 2007[321]		48 wk	45	RCT, Parallel Arm	eGFR 18.3±4.6	55±12	17/10	excl	NR	VLPD	30	0.3	veg.	Y	NR
					eGFR 17.9±4.3	54±11	15/11			LPD	30	0.6	NR	N	
Chauveau 2003[322]		24 mo	13	Single Arm	eGFR 15±4.7	55±12	8/5	NR	NR	VLPD	35	0.3	veg.	Y	5-7[e]
Di Iorio 2003[323]		24 mo	10	Parallel Arm	CrCl 15.4±4.2	57±17	12/8	NR	NR	VLPD	35	0.3	veg.	Y	NR
			10		CrCl 17.3±3.5	52±15				LPD	35	0.6	NR	N	
Chauveau 1999[324]		12 mo	10	Single Arm	eGFR 13.2±4.8	57.1±9.3	6/4	NR	NR	VLPD	35	0.3	veg.	Y	5-7[e]
Lafage-Proust 1999[b325]		65±24.4 mo	16	Single Arm	eGFR 14.6±4.5	53.4±17.3	8/8	0	NR	VLPD	35	0.4	veg.	Y	7-9[e]
Barsotti 1996[b326]		12.8±5.7 mo	22	Two Single Arms	CrCl 20-40	20-60	7/4	excl	NR	LPD, Mixed to Veg.	>35	0.7	veg.	N	10.5[e]
		14.1±5.0 mo				18-54	8/3			FD to LPD Veg.	>35	0.6	veg.	N	8[e]
Combe 1995[327]		24 mo	29	Single Arm	eGFR 13.7±4.5	56.1±13.4	20/9	NR	NR	VLPD	35	0.4	veg.	Y	5-7[e]
Combe 1993[328]		23.0±10.	27	Single	eGFR: 15.59±5.41	53.3±	28/	NR	NR	VLPD-C	35	0.3	veg.	Y	3-5[e]

	6 mo		Arm		14.6	12									
	23.7±11.6 mo	13							VLPD - NC						
Aparicio 1992[329]	19.2±7.6 mo	27	Single Arm						VLPD - C						
	19.9±8.1 mo	13							VLPD - NC						
Barsotti 1992[330]	8-58 mo	23	Single Arm	CrCl 24.1±19.8	24-69	16/7	23	NR	VLPD	30-35	0.35	veg.	Y	350-400
									LPD	30-35	0.7	veg.	N	600-700
Lafage 1992[331]	12 mo	17	Single Arm	eGFR 13.2±4.6	53.5±12.5	14/3	excl	NR	VLPD	35	0.3	veg.	Y	300-500
Aparicio 1991[332]	12 mo	20	Single Arm	eGFR 14.9±6.2	54.1±14.8	15/5	NR	NR	VLPD	35	0.3	veg.	Y	300-500
Aparicio 1990[333]	≥ 18 mo	66	Single Arm	CrCl: VLPD-C 17.2±15.6 VLPD-NC 20.7±10.3	48.6±29.4	52/43	NR	NR	VLPD	35	0.3	veg.	Y	3-5[e]
Di Landro 1990[b334]	36 mo	69	RCT, Parallel Arm	PCr 4.3±1.8	45±17	NR	NR	NR	VLPD	35	0.3	mostly veg.	Y	4[e]
				PCr 4.3±1.3	48±12				LPD	35	0.6	NR	N	10[e]
Ciardella 1988[b335]	13.6±6.9 mo	11	Single Arm	CrCl 43.4±9.6	18-61	8/3	11	NR	VLPD	35	0.3	veg.	Y	3.5[e]
	15.1±5.2 mo								FD	NR	1.2	NR	N	NR
Barsotti 1988[336]	17.4±5.8 mo	8	Parall	CrCl 19.2±13.4	44.7±11.8	5/3	8	NR	VLPD	35	0.25-0.35	veg.	Y	4.5-5.5[e]

				Arm						LPD	35	0.5 - 0.6	veg.	Y	8-12[e]

Table 4.1. Summary of study characteristics

Values are shown as mean and standard deviation or ranges unless otherwise specified. Black fill with white text indicates the five studies included in the plant-protein intervention subset. Combe et al. (1993)[328] was the follow up study to Aparicio et al. (1992)[329] in which the same participants were followed for an average of ~4 additional months following completion of the first study. Thus, the same study characteristics were reported in each study and combined in the above table. *CrCl* creatinine clearance, *eGFR* estimated glomerular filtration rate, *SCr* serum creatinine, *PCr* plasma creatinine, *excl* excluded, *NR* not reported, *C* Caucasian, *H* Hispanic, *AA* African American, W white, *VLPD* very low protein diet, *VLPD-C* very low protein diet compliant, *VLPD-NC* very low protein diet noncompliant, *LPD* low protein diet, *MD* Mediterranean diet, *FD* free diet, *UPD* usual protein diet, *UC* uncontrolled, *veg*. vegetable, *Y* yes, *N* no, *M* male, *F* female, *Suppl* supplementation

[a]Values for age, sex, and diabetes are representative of the entire population as exact values for the 65 participants of interest were not available. [b]Units for outcomes were not reported. [c]Values reported as median {95% CI} [d]kcal/d [e]mg/kg/d, [f]median, [g]g/d

Figure 4.3. Risk of bias assessment

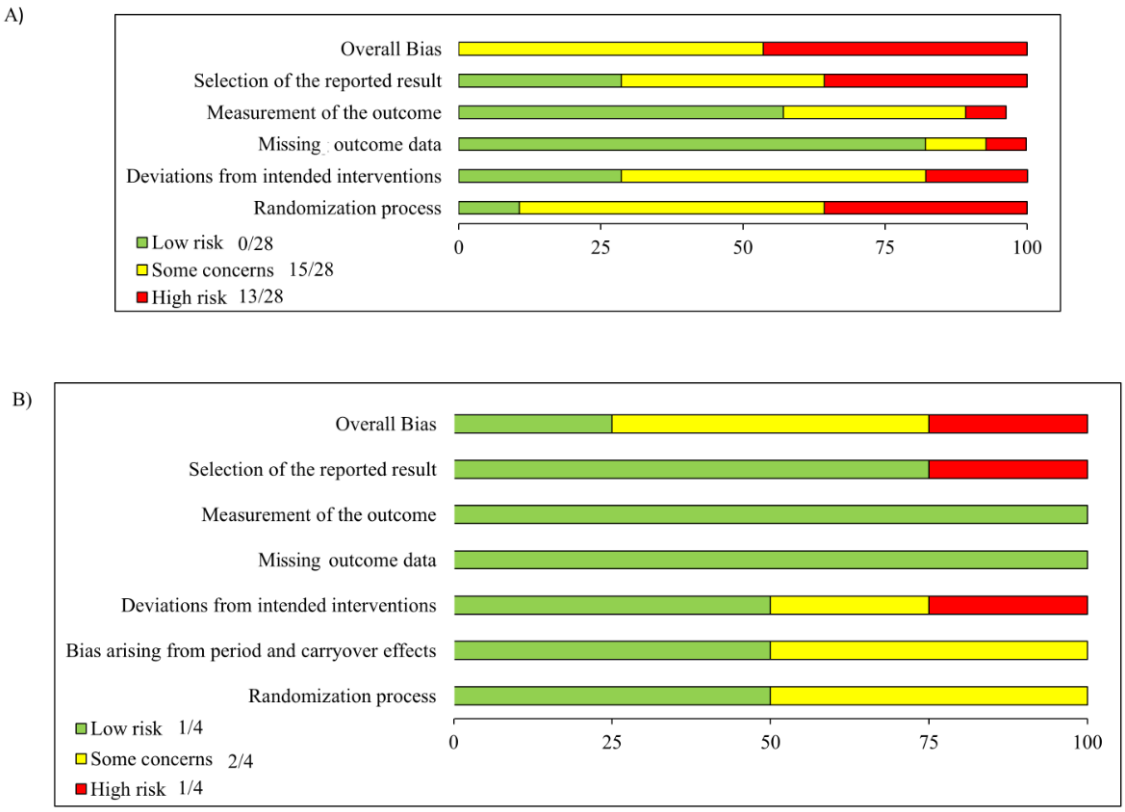

Figure 4.3. Risk of bias assessment
Risk of bias was assessed using the ROB-2 tool. **A) Risk of bias assessment of randomized controlled trials.** Of the 28 studies assessed, overall risk of bias was either moderate (53.6%) or high (46.4%). **B**) **Risk of bias assessment of crossover trials.** Of the 4 studies assessed, overall risk of bias was low (25%), moderate (50%), and high (25%).

Table 4.2. Summary of kidney function outcomes (Subset of five studies with only protein source intervention)

Study	Study Duration Category	Length	N	Treatment	Direction of change			
					CrCl or eGFR (mL/min/1.73m^2)	Serum Creatinine (mg/dL)	Serum BUN or Urea (mg/dL)	Proteinuria (g/24 hr)
Moorthi 2014[305]	Short term (1-4 weeks)	4 wks	13	70% plant prt E vs B	⬌	⬇	NR	NR
Moe 2011[307]		1 wk/diet	8	Veg. vs meat	⬌	NR	NR	NR
Fois 2019[308]	Mid-length (>4-26 weeks)	≥3 mo	65	LPD veg. vs LPD mix	⇔	⬌	⇔	⇔
Soroka 1998[313]		6 mo	9	Veg. vs mix	⬌⬌⬌	⬌	⬇⬇⬇	⬌
Barsotti 1996[326]	Long term (>26 2weeks)	12.8 ± 5.7 mo	11	LPD mixed to LPD veg.	⇩	⇔	⇔	NR

Table 4.2. Summary of kidney function outcomes (Subset of five studies with only protein source intervention)

Direction of change for CrCl or eGFR, serum creatinine and BUN or urea, and proteinuria is shown for the subset of studies where change in protein source from animal to plant was the main intervention. For all outcomes, mixed results were observed across studies, except for proteinuria, where no change was observed. Horizontal arrows indicate no change, up arrows indicate an increase, and down arrows indicate a decrease in the outcome reported. Effect size is shown by arrow number with one arrow representing a small magnitude of effect, two arrows representing a medium magnitude of effect, and three arrows representing a large magnitude of effect. White arrows were used when effect size was unable to be determined. *NR* not reported, *prt* protein, *E* endpoint, *B* baseline, *LPD* low protein diet, *veg* vegetarian

Table 4.3. Summary of CKD-MBD outcomes (Subset of five studies with only protein source intervention)

Study	Study Duration Category	Length	N	Treatment	Direction of change			
					Serum P (mg/dL)	Serum PTH (pg/mL)	Serum Ca (mg/dL)	Urinary P (mg/24 hr)
Moorthi 2014[305]	Short term (1-4 weeks)	4 wks	13	70% plant prt E vs B	⟷	⟷	⟷	↓↓↓
Moe 2011[307]		1 wk/diet	8	Veg. vs meat	↓↓↓	[b] ↓	⇄	⇄
Fois 2019[308]	Mid-length (>4-26 weeks)	≥3 mo	65	LPD veg. vs LPD mix	NR	⇔	NR	NR
Soroka 1998[313]		6 mo	9	Veg. vs mix	⇄	NR	⇄	↓↓↓
Barsotti 1996[326]	Long term (>26 weeks)	12.8 ± 5.7 mo	11	LPD mixed to LPD veg.	⇔	NR	NR	NR

Table 4.3. Summary of CKD-MBD outcomes (Subset of five studies with only protein source intervention)

Direction of change for serum P, PTH, and Ca, and urinary phosphorus is shown for the subset of studies where change in protein source from animal to plant was the main intervention. Mixed results were observed across studies for serum P and PTH, but consistent results were observed for serum calcium and urinary P. Horizontal arrows indicate no change, up arrows indicate an increase, and down arrows indicate a decrease in the outcome reported. Effect size is shown by arrow number with one arrow representing a small magnitude of effect, two arrows representing a medium magnitude of effect, and three arrows representing a large magnitude of effect. White arrows were used when effect size was unable to be determined. *NR* not reported, *prt* protein, *E* endpoint, *B*

baseline, *LPD* low protein diet, *veg* vegetarian [b]Decreased in meat-based group compared with vegetarian group.

Chapter 5: Discussion and Future Direction

Summary and Synthesis

Acute High Dietary Phosphorus Following Low Phosphorus Diet Acclimation Does Not Enhance Intestinal Fractional Phosphorus Absorption in Nephrectomized Male Rats

In this rodent study, we performed an *in vivo* oral gavage absorption test using ^{33}P. Our findings suggest that an acute high phosphorus load after consuming a low phosphorus diet, mimicking a bout of non-adherence, does not lead to enhanced intestinal fractional phosphorus absorption in 5/6 nephrectomized male rats, at least in the short term. This was accompanied by lower gene expression of NaPi-2b in the jejunum of rats kept on the LPHP compared with the HP diet and may be one reason why intestinal phosphorus absorption efficiency was not enhanced in the LPHP group. Interestingly, we also found no difference in plasma phosphorus between the LPHP and HP groups, but both groups had higher plasma phosphorus than the LP group.

Our findings are contrary to the findings by another group[39] who investigated the response to differing phosphorus loads using an *in vitro* phosphorus uptake technique from isolated brush border membranes of healthy male rats. However, our study was unique in that it was performed *in vivo* rather than *in vitro*. This allows for the gastrointestinal tract to remain completely intact and is likely to lead to a more physiologically relevant response to a phosphorus load rather than an *in vitro* system. In fact, many studies available on phosphorus

absorption are performed *in vitro*[34,43,230,231] with little data available using *in vivo* techniques. However, our findings and the findings[30,59] of others suggest that *in vitro* techniques may not be replicated *in vivo*.

Phosphorus Bioaccessibilty of Emerging Processed Pulse and Soy-Based Protein Products by In Vitro Simulation of Human Digestion

Our pilot investigation of phosphorus bioaccessibility in emerging processed pulse and soy-based protein products by *in vitro* simulation of human digestion suggests that plant-based protein products, as alternatives to animal-based products may be suitable for inclusion into the diets of patients with CKD. Of the 33 soy and pulse-based protein products evaluated, most offered lower bioaccessible phosphorus per 100g serving than their animal comparators. However, pulse-based milk alternatives had >2.5x the absolute bioaccessible phosphorus in comparison to cow's milk and soy-based milk alternatives. In contrast, we found that average phosphorus bioaccessibility for soy-based milk alternatives were comparable to cow's milk and offered a more optimal bioaccessible phosphorus-to-protein ratio. This finding is of particular importance for patients with CKD on dialysis who are recommended to follow a high protein but low phosphorus diet[121] as consuming soy-based milk may optimize their protein intake while minimizing their phosphorus consumption.

We also observed a wide range of phosphorus bioaccessibility among plant-based protein products ranging from an average of ~32% in pulse-based beef to as high as ~103% in pulse-based milk. Numerous factors could contribute

to the wide variation in phosphorus bioaccessibility of these products such as differences in food matrices, processing techniques, and cooking methods.[340] In addition, our data indicate that the presence of at least one phosphate additive in a food product does not necessarily translate to 100% bioaccessible phosphorus, which has also been observed by others.[67] These data highlight the need for greater determination and quantification of bioaccessible phosphorus in a variety of products and meals to better inform individualized dietary guidance for patients with CKD.

Effects of Plant-Based Protein Consumption on Kidney Function and Mineral Bone Disorder Outcomes in Adults with Stage 3-5 Chronic Kidney Disease: A Systematic Review

We conducted a systematic review to determine the effect of plant-based protein on kidney function and mineral bone disorder outcomes in adults with stage 3-5 CKD not on dialysis. Overall, there was limited evidence from intervention studies on this topic. In fact, although 32 studies met inclusion criteria, only five studies investigated the independent effect of a change in protein source between animal and plant-based protein. Of all included studies, most were of suboptimal methodological quality and reported inconsistent findings across kidney function and CKD-MBD outcomes. Additionally, heterogeneity in study design, duration, and interventions hindered our ability to perform a meta-analysis.

We found that the most widely reported outcomes were CrCl or eGFR, serum BUN, phosphorus, and PTH. Thus, this systematic review underscores the need for greater reporting of CKD-MBD markers (e.g., FGF-23, 1,25OHD) and hard endpoints (e.g., cardiovascular and fracture events). Of the 5 included studies, only one long term study found a worsening of kidney function with a vegetarian low protein diet, but this was a single arm study, which limits our ability to determine if the change in kidney function was attributed to the diet intervention or just a natural decline over time. Similarly, for CKD-MBD outcomes, only one short duration study found a decrease in serum phosphorus. Notably, the strength of the study design (i.e., randomized, crossover, controlled feeding) demonstrates the efficacy of a low phosphorus diet in the short term but its effectiveness in the long term remains to be known.

Strengths and Limitations

Each of the above-mentioned studies has strengths and limitations that were discussed in each individual chapter. For example, a limitation to the rodent study was that only one bout of non-adherence was tested. It could be that a different effect may have been observed for phosphorus absorption and CKD-MBD markers after multiple bouts of non-adherence, but we are unable to draw these conclusions. Others have shown that multiple bouts of non-adherence to a low phosphorus diet can lead to vascular calcification in rodent models.[247,248] However, more research is needed to understand how these observations translate to humans. Another limitation of the rodent study was that the model of

CKD we used was more similar to the observations seen during the early onset of the disease rather than late-stage. As kidney disease progresses, abnormalities in mineral metabolism become more pronounced[107] and could be more severely affected by bouts of non-adherence to a low phosphorus diet than what is observed in earlier stages. Further, limitations include the use of only male rats and measuring only gene expression of the phosphate transporters. However, there were many strengths to this study. As mentioned above, using the *in vivo* oral gavage technique is a major strength of this study as the physiology of the gastrointestinal tract remained intact. Moreover, another strength was the study design as efforts were made to reduce risk of bias through randomizing and concealing allocation groups until the study diets and experimentation period began.

There are also several limitations to our study of phosphorus bioaccessibility. For example, only one sample of each product was purchased, so we were not able to determine within sample variation. Within sample variation may exist between product lots and/or manufacturing locations. Phosphorus content of the soil can also vary by geographic location[281,282,341] and may impact phosphorus content of the soy and pulse ingredients used in these products. Therefore, further studies are needed to understand within product variation. An additional limitation is that an *in vitro* method was used to determine phosphorus bioaccessibility which may not reflect bioaccessibility *in vivo* as individual people have different efficiencies for making phosphorus accessible for

absorption. Other limitations include the use of a static rather than dynamic model for simulating human digestion and our inability to distinguish between phosphate salts. However, there are numerous strengths that should be acknowledged. For example, the *in vitro* digestion experiments were performed based on an international consensus, standardized protocol.[262] Therefore, we are confident our methods accurately simulate gastrointestinal digestion of adult humans and are directly applicable to this population. Another strength was that a wide variety of products were tested and can be used to generate hypotheses to drive future work in this area.

One limitation of our systematic review is that it was narrowly focused to determine the effects of plant-based protein interventions. Plant-based protein is only one component of a plant-based diet. Thus, data may be available more generally on the effects of an overall plant-based diet on kidney function and CKD-MBD outcomes in this population. Additionally, we only focused on kidney function and CKD-MBD outcomes, which encompass only a fraction of the potential health outcomes that could be affected by consuming greater plant-based protein or a plant-based diet. Other potential health outcomes that could be affected by this diet include metabolic acidosis, inflammation, and uremic retention solutes.[251] However, there are many strengths to this systematic review including the extensive search strategy and categorizing of studies by duration. As described above, the heterogeneity of the available studies prevented us from performing a meta-analysis, but categorizing the studies by duration allowed us

to synthesize and make interpretations on how plant-based protein interventions may affect kidney function and CKD-MBD outcomes over time. Additionally, this systematic review highlights the limitations and gaps in the currently available literature. Much of the literature was of suboptimal methodological quality, performed over 2 decades ago, and was primarily conducted to investigate a change in protein amount which happened to be accompanied by a change in protein source. Therefore, these findings can be used to drive future work in this area.

Future Direction

The data presented in this dissertation adds foundational knowledge to our understanding of phosphorus management in CKD. However, more research is needed to optimize dietary phosphorus recommendations for patients with CKD. As mentioned previously, reducing phosphorus intake is difficult and can lead to non-adherence to these dietary recommendations. Although we show that a bout of non-adherence does not affect intestinal fractional phosphorus absorption in rats, there could be other potential harms of deviating from a low phosphorus diet. This makes it critically important to understand how dietary phosphorus restriction or bouts of non-adherence to this recommendation affect clinical endpoints (e.g., cardiovascular disease, fracture risk, etc.). Further, strategies must be put in place to reduce the burden and difficulty of following a low phosphorus diet.

One such approach recommended by KDOQI and KDIGO is to take phosphate source into consideration, but data in this area are sparse. Specifically, in our systematic review, we found that available evidence for the effect of plant-based protein consumption on kidney function and CKD-MBD outcomes was heterogeneous and of suboptimal methodological quality. Thus, underscoring the need for future research in this area. There must be a call to action for researchers to conduct rigorous intervention studies that reduce risk of bias, report on a greater number of CKD-MBD outcomes, and directly investigate the effect of protein or phosphate source on health outcomes in CKD. These studies could also be used to investigate the potential harms or risk of consuming greater plant-based protein in CKD. Our data on phosphorus bioaccessibility shows that plant-based protein alternatives may be suitable for inclusion into the diets of patients with CKD. However, this data is hypothesis generating and research on this topic must be continued. Future research should be conducted that focuses on understanding within product variation, phosphorus bioaccessibility from mixed meals, and the relationship between *in vitro* phosphorus bioaccessibility and phosphorus bioavailability *in vivo*.

Conclusion

Overall, there are many facets to consider when recommending a reduced phosphorus diet to patients with CKD. The strict nature of such a diet can be challenging to adhere to and may lead to unnecessarily restricting groups of foods thought to be part of a healthy eating pattern. While a bout of non-

adherence may impact other health outcomes, we show that a single bout of non-adherence has no effect on intestinal fractional phosphorus absorption in nephrectomized male rats. However, more research must be done to understand how this translates to humans. Further, as interest in plant-based protein and plant-based diets continues to grow, this could be utilized as a dietary approach to lessen the stress and difficulty of following a low phosphorus diet. We show that of a subset of emerging processed pulse and soy-based protein products, most offered lower bioaccessible phosphorus per 100g serving. We also show that limited evidence from intervention studies is available to determine the effect of plant-based protein consumption on kidney function and CKD-MBD outcomes in adults with stage 3-5 CKD not on dialysis These data emphasize the need to continue to conduct research in this area to inform and optimize dietary phosphorus recommendations for patients with CKD to improve CKD-MBD symptoms and overall quality of life.

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Appendix A: Supplementary Information - Chapter 2: Acute High Dietary Phosphorus Following Low Phosphorus Diet Acclimation Does Not Enhance Intestinal Fractional Phosphorus Absorption in Nephrectomized Male Rats

Table A.A.S1. Study diet formula

	TD.85010 (LP, 0.1% P) (g/kg)	TD.85349 (HP, 1.2% P) (g/kg)
Egg White Solids	200	200
Sucrose	525.2	501.2
Corn Starch	150	150
Corn Oil	50	50
Cellulose	20	20
Mineral Mix, Ca-P Deficient (79055)	13.4	13.4
Calcium Carbonate	14.7	5.03
Potassium Bicarbonate	8.1	-
Sodium Chloride	4.7	-
Sodium Phosphate, Monobasic, Monohydrate	1.7	12.9
Potassium Phosphate, Monobasic	1.7	12.7
Calcium Phosphate, Monobasic, Monohydrate	-	24.4
Vitamin Mix, Teklad (40060)	10	10
Biotin	0.004	0.004
Yellow Food Color	0.3	0.3
Blue Food Color	0.15	-
Red Food Color	-	0.15
Protein (% kcal from)	17.2	17.6
Carbohydrate (% kcal from)	70.8	70.1
Fat (% kcal from)	12	12.3
Kcal/g	3.8	3.7

Table A.A.S1. Study diet formula
Study diets were formulated to contain either low (0.1% P w/w, TD.85010, Envigo Teklad, Indianapolis, IN, USA) or high phosphorus (1.2% P w/w, TD.85349 Envigo Teklad, Indianapolis, IN, USA).

Table A.A.S2. Day 7 food consumption and final body weight

	Health Status		Diet			Health Status x Diet						ANCOVA P-Values			
						LP		LPHP		HP					
	Sham	CKD	LP	LPHP	HP	Sham	CKD	Sham	CKD	Sham	CKD	Model	Health Status	Diet	HxD
Day 7 Food Consu-mption (g/d)[1]	17 (4.34)	19 (4.32)	17 (4.32)	17 (4.32)	20 (4.38)	17 (4.31)	17 (4.16)	15 (4.31)	19 (4.16)	19 (4.5)	20 (4.31)	0.25	0.12	0.12	0.24
Final Body Weight (g)[1]	371 (27.3)*	340 (27.2)	347 (27.3)	361 (27.3)	358 (27.3)	362 (27.2)	333 (27)	369 (27.2)	353 (27)	381 (27.3)	335 (27.2)	**0.0005**	**<0.0001**	0.21	0.18

Table A.A.S2. Day 7 food consumption and final body weight
ANCOVA p-values for the overall model (P_{Model}), main of effect of health status (P_{Health}), diet (P_{Diet}), and their interaction (P_{HxD}) for day 7 food consumption and final body weight. LS means ± SD are shown. Food consumption did not differ between groups. Final body weight was lower in CKD compared to sham rats, as expected due to CKD.
[1]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Figure A.A.S1. Oral dose curves

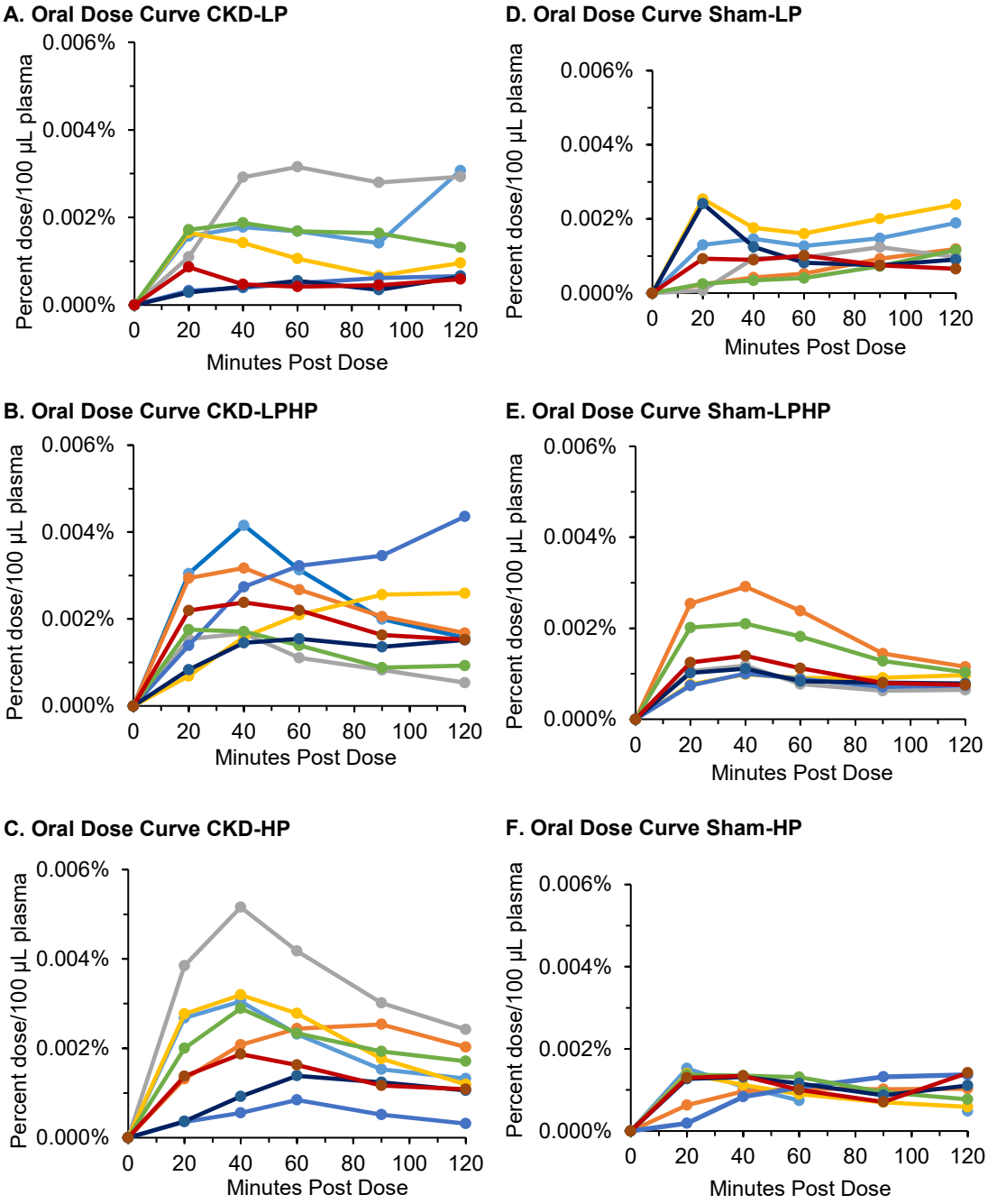


Figure A.A.S1. Oral dose curves
Plasma ^{33}P levels at each timepoint are shown as a percent of oral isotope dose for each individual rat. Each color per graph represents one rat. Values presented are from 100 µL of plasma counted at each time point over a 2-hour period. Axes for all graphs were kept consistent for ease of interpretation.

n= 7 sham LP, 7 sham LPHP, 6 sham HP, 7 CKD LP, 8 CKD LPHP, 8 CKD HP

Figure A.A.S2. I.V. dose curves

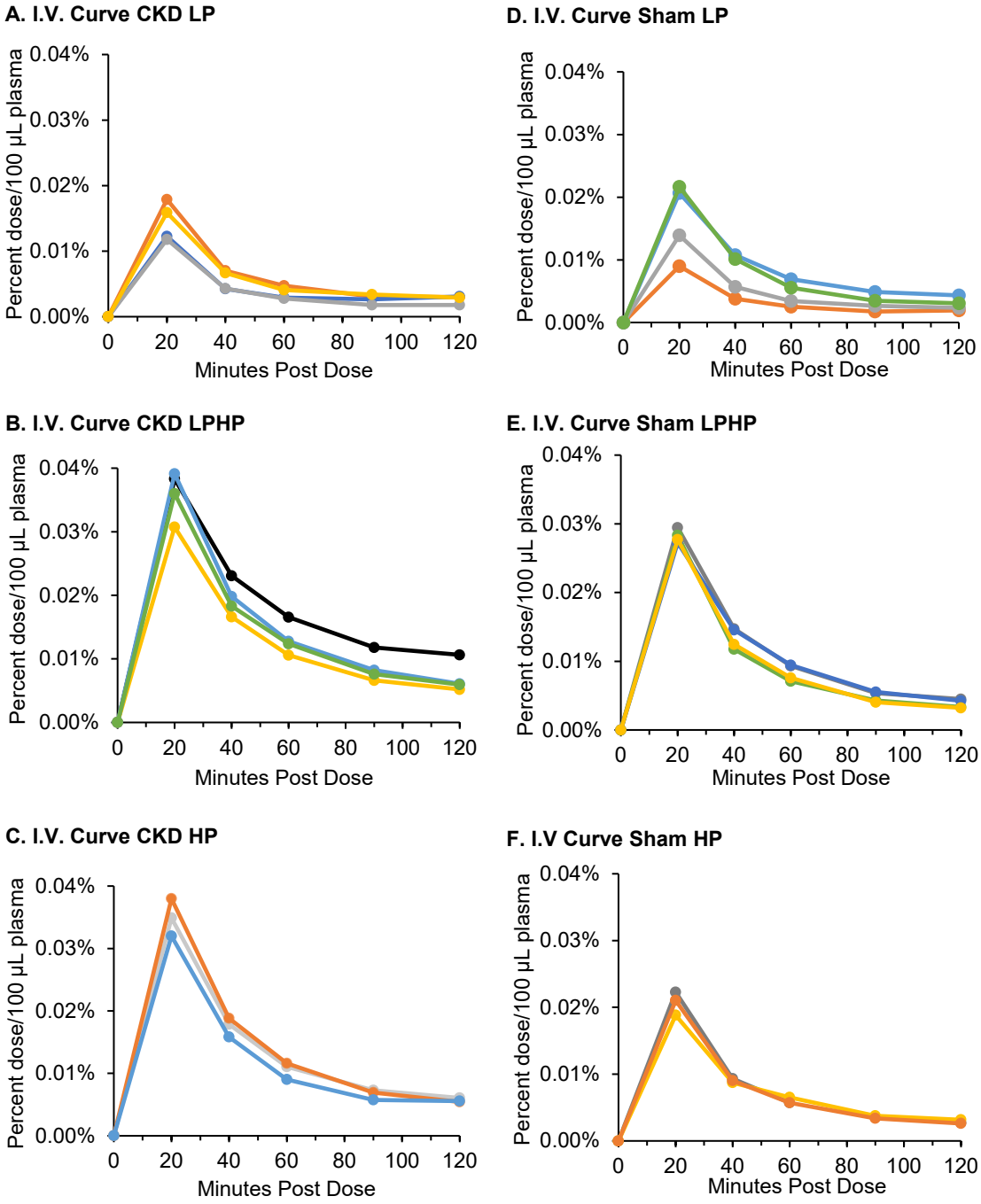


Figure A.A.S2. I.V. dose curves

Plasma ^{33}P levels at each timepoint are shown as a percent of I.V. isotope dose for each individual rat. Each color per graph represents one rat. Values presented are from 100 µL of plasma counted at each time point over a 2-hour period. Axes for all graphs were kept consistent for ease of interpretation.
n= 4 sham LP, 4 sham LPHP, 3 sham HP, 4 CKD LP, 4 CKD LPHP, 3 CKD HP

Table A.A.S3. Intestinal fractional phosphorus absorption and endpoint plasma biochemistries by health status and diet treatment

	Health Status		Diet			ANCOVA P-Values			
	Sham	CKD	LP	LPHP	HP	Model	Health Status	Diet	HxD
Intestinal Fractional Phosphorus Absorption (AUC_{PO}/AUC_{IV})[1]	0.09 (0.04)	0.11 (0.05)	0.14 (0.04)	0.09 (0.04)	0.09 (0.04)	0.12	0.31	**0.03**	0.97
BUN (mg/dL)[2]	22.5 (5.12)	38.9 (5.09) *	31.8 (5.13)	30.6 (5.13)	29.7 (5.14)	**<0.0001**	**<0.0001**	0.40	0.73
P (mg/dL)[3]	9.7 (1.17)	10.4 (1.15)	5.8 (1.15)	12.5 (1.13)	12.0 (1.13)	**<0.0001**	**0.02**	**<0.0001**	**<0.0001**
Ca (mg/dL)[4]	10.7 (1.15)	10.6 (1.12)	11.3 (1.15) [a]	9.8 (1.17) [b]	11.0 (1.16)[a]	**<0.0001**	0.71	**0.0001**	0.08
iFGF23 (pg/mL)[5]	242 (93.1)	403 (139)	153 (51.3)	380 (142.6)	523 (177.8)	**<0.0001**	**<0.0001**	**<0.0001**	**0.006**
iPTH (pg/dL)[6]	1456 (642.5)	1355 (633.6)	707 (634) [c]	1337 (634)[b]	2174 (642.1)[a]	**<0.0001**	0.53	**<0.0001**	0.12
$1,25(OH)_2D_3$(pg/mL)[7]	147 (50.3)	145 (50.1)	138 (50.5)	149 (50.2)	151 (50.5)	0.53	0.87	0.68	0.51

Table A.A.S3. Intestinal fractional phosphorus absorption and endpoint plasma biochemistries by health status and diet treatment
LS means ± SD for intestinal fractional phosphorus absorption and plasma biochemistries by health status and diet treatment. *represents <0.0001 between health status. Different superscripted letters represent group differences.
[1]n= 7 sham LP, 7 sham LPHP, 6 sham HP, 7 CKD LP, 8 CKD LPHP, 8 CKD HP
[2]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP
[3]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 11 CKD LPHP, 10 CKD HP
[4]n= 11 sham LP, 10 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP
[5]n= 11 sham LP, 10 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 9 CKD HP

[6]n= 11 sham LP, 11 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP
[7]n= 9 sham LP, 10 sham LPHP, 9 sham HP, 11 CKD LP, 12 CKD LPHP, 11 CKD HP

Table A.A.S4. Intestinal phosphate transporter gene expression by health status and diet treatment

	Health Status		Diet			ANCOVA P-Values			
	Sham	CKD	LP	LPHP	HP	Model	Health Status	Diet	HxD
Duodenal NaPi-2b/RPLP0[1]	2.10 (2.12)	1.96 (1.87)	2.13 (1.68)	1.80 (1.31)	2.19 (1.43)	0.27	0.72	0.71	0.17
Duodenal Pit-1/RPLP0[2]	1.10 (0.61)	1.04 (0.58)	1.02 (0.58)	1.04 (0.58)	1.14 (0.65)	0.49	0.66	0.82	0.0501
Duodenal Pit-2/RPLP0[a1]	1.11 (0.39)	1.12 (0.35)	0.9 (0.38)[b]	1.37 (0.38)[a]	1.07 (0.36)[b]	**0.01**	0.91	**0.0002**	0.64
Jejunal NaPi-2b/RPLP0[3]	2.61 (2.53)	2.36 (2.33)	2.92 (2.98)[a,b]	1.46 (1.39)[b]	3.60 (3.62)[a]	**0.007**	0.69	**0.01**	0.18
Jejunal Pit-1/RPLP0[4]	2.36 (2.05)	2.77 (2.48)	2.80 (2.53)	2.1 (1.92)	2.89 (2.53)	0.33	0.47	0.41	0.16
Jejunum Pit-2/RPLP0[5]	1.60 (0.71)	1.39 (0.59)	1.46 (0.62)	1.55 (0.67)	1.46 (0.65)	**0.0003**	0.19	0.86	0.98

Table A.A.S4. Intestinal phosphate transporter gene expression by health status and diet treatment

LS means ± SD for intestinal phosphate transporters gene expression by health status and diet treatment. Different superscripted letters represent group differences.

[1]n = 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 11 CKD LPHP, 11 CKD HP

[2]n= 11 sham LP, 11 sham LPHP, 9 sham HP, 12 CKD LP, 12 CKD LPHP, 10 CKD HP

[3]n= 10 sham LP, 11 sham LPHP, 8 sham HP, 11 CKD LP, 12 CKD LPHP, 11 CKD HP

[4]n= 10 sham LP, 10 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

[5]n= 11 sham LP, 10 sham LPHP, 8 sham HP, 12 CKD LP, 12 CKD LPHP, 11 CKD HP

Appendix B: A Journey Through Method Development: Determination of Bioaccessible Phosphorus Content via Simulation of Human Digestion and Subsequent Dialysis

Background:

Known:

1. Taking phosphate source into consideration when making dietary recommendations for patients with CKD is a recently championed approach to limit dietary phosphorus intake.
2. Sparse data is available for bioavailable or bioaccessible phosphorus of food sources.

Unknown:

1. What method should be utilized to digest food products?
2. What is the most appropriate dialysis membrane/device, molecular weight cut off (MWCO), and dialysis timing to utilize for a critical separation of dialysis (i.e., dialyze freely available phosphorus and retain phytic acid)?

Aim: Undertake extensive method development to optimize a protocol to accurately determine phosphorus bioaccessibility of food products.

Question 1: What method should be utilized to digest food products?

Numerous protocols are available that simulate human gastrointestinal digestion. However, we chose to utilize the INFOGEST method for our *in vitro* digestion experiments.

Box 1. What is the **INFOGEST** method of human digestion?

INFOGEST is an international network of excellence on the fate of food in the gastrointestinal tract. One of three main scientific goals for this initiative is aimed at promoting the harmonization of currently used digestion models. Thus, the protocol developed by this network is an international consensus method that is validated and standardized to accurately simulate human gastrointestinal digestion of adults. Briefly, this consists of an oral, gastric and intestinal phase that were specifically developed to mimic the physiology of human digestion.

Question 2: What is the most appropriate dialysis membrane/device, molecular weight cut off (MWCO), and dialysis timing to utilize for a critical separation of dialysis (i.e., dialyze freely available phosphorus and retain phytic acid)?

Numerous varieties of dialysis membranes/devices with varying MWCOs are available to perform dialysis techniques. Therefore, we performed experiments to determine the most appropriate dialysis membrane to choose for the determination of bioaccessible phosphorus.

Box 2. What are the molecular weights of the molecules we are trying to dialyze or retain in the membrane?

Phosphate: 94.97 g/mol

Phytic acid: 660.04 g/mol

Test 1: Perform the *in vitro* digestion and subsequent dialysis test based on our understanding of the methods used by Karp et al.[216,217]

Test 1 Methods:

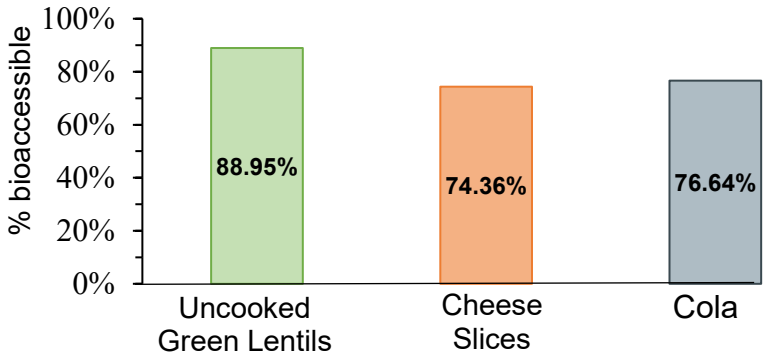
1. Perform *in vitro* digestion experiments using INFOGEST protocol of products with varying food matrices (uncooked green lentils, cheese slices, cola).
2. Following completion of digestion dilute digesta to a known volume of 25 mL.
3. Pipet 6 mL of digesta into standard flat dialysis membrane with a MWCO of 3500 D (Repligen 086705A) and clip on both ends with dialysis clips.
4. Dialyze diluted digesta against 45 mL of double deionized water (DDI) overnight (~16 hours) at room temperature.
5. Prepare dialysate in 2.8% nitric acid solution to be analyzed for phosphorus content via inductively coupled plasma atomic emission spectroscopy (ICP-OES).

Test 1 Results: As expected, percent bioaccessible phosphorus for cola was ~77% and for cheese slices was ~74% which was similar to the findings by Karp et al.[216,217] for cola and processed cheese. The percent bioaccessible phosphorus for uncooked lentils was ~89%, which was much higher than the findings by Karp et al. for uncooked lentils (30%). It is possible that cooking processes could drastically impact bioaccessible phosphorus content, but the

lentils we tested, and the lentils tested by Karp et al.[216] were not cooked prior to testing. Thus, the bioaccessible phosphorus of these products should be relatively comparable. Further questions that arose from this test: **(1) Is it possible that a 3500 MWCO is too large to retain phytic acid? (2) Is the dialysis membrane working as intended?**

Test 1 Figures:

Figure A.B.1. Test 1 In vitro digestion and subsequent dialysis 3500 D MWCO



Test 2: Perform dialysis of sodium phytic acid and sodium phosphorus solutions to determine if phytic acid is being retained and phosphorus is freely dialyzing.

Test 2 Methods:

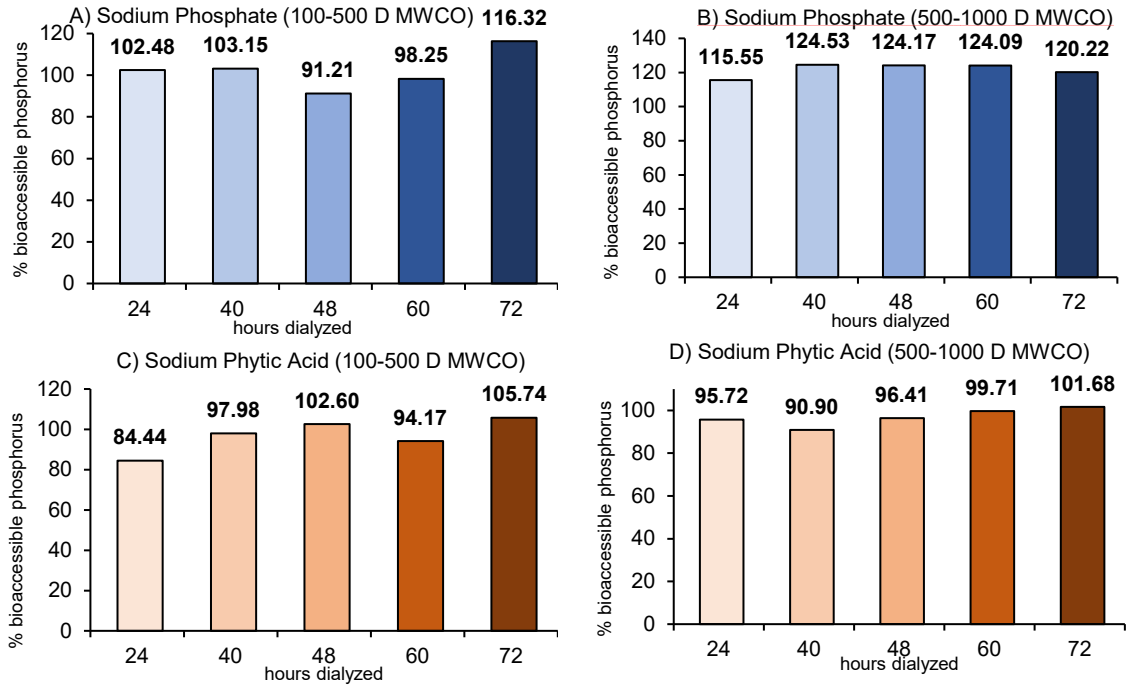
1. Sodium phytic acid solution: Weigh 3g of sodium phytic acid from rice (Sigma-Aldrich P8810) and add to a 50 mL volumetric flask. Dilute to 50 mL with DDI water and invert to mix.
2. Sodium phosphate dibasic solution: Weigh 7g of sodium phosphate dibasic (Fisher Scientific S375) and add to a 50 mL volumetric flask. Dilute to 50 mL with DDI water and invert to mix.
3. Adjust the pH of each solution as appropriate to near normal (pH 7) using 5M NaOH.
4. Pipet 5 mL of each solution into separate dialysis membranes with a 100-500 D MWCO (Repligen, 131054) and clipped on both ends.
5. Pipet 5 mL of each solution into separate dialysis membranes with a 500-1000 D MWCO (Repligen, 131090) and clipped on both ends.

6. Dialyze the sodium phytic acid and sodium phosphate solutions against 45 mL of DDI water for 24, 40, 48, 60, and 72 hours.
7. Prepare dialysate in 2.8% nitric acid solution to be analyzed for phosphorus content via microwave plasma atomic emission spectroscopy (MP-AES).

Test 2 Results: At all timepoints the dialysate of the sodium phosphate solution was similar to or above 100%. Over the 24-72 hours, the percent bioaccessible phosphorus ranged from 91-116% after dialyzing with the 100-500 D MWCO and 115-124% after dialyzing with the 500-1000 D MWCO. Similar results were observed for the dialysate of the sodium phytic acid. Over 24-72 hours, the percent bioaccessible phosphorus ranged from 84-105% after dialyzing with the 100-500 D MWCO and 90-102% after dialyzing with the 500-1000 D MWCO.

Test 2 Figures:

Figure *A*.B.2. Test 2 Sodium phytic acid and sodium phosphate dialysis



Test 3: Visually determine if the dialysis membranes are working as intended.

Box 3. How could we visually determine if our dialysis membranes were leaking?
Brilliant Blue has a similar molecular weight (792.85 g/mol) as phytic acid (660.04 g/mol) and due to its color, would be visible in the dialysate if the membrane was leaking.

Test 3 Methods:

1. Create a 0.5% w/v Brilliant Blue (Sigma-Aldrich B1131) and water solution.
2. Pipet 5 mL of the prepared solution into 3 different membranes with a MWCO of 500-1000 D.
    a. Membrane 1: Clip the dialysis membrane on each end, as previously described above.
    b. Membrane 2: Fold the dialysis membrane and clip on each end.
    c. Membrane 3: Use a dialysis device (Float-a-lyzer® G2, Repligen G235051).
3. Dialyze each membrane type against 45 mL of ultrapure water for up to 72 hours.
4. Visually inspect dialysate for the presence of Brilliant Blue.

Test 3 Results: The dialysate of all membranes remained free of the Brilliant Blue solution for 6 hours (Figure 3A-C). However, after 8 hours, the Brilliant Blue solution had seeped into the dialysate of membrane 1. The dialysate of membrane 2 and 3 remained clear at 8 hours (Figure 3D-F). Following 24 hours, the Brilliant Blue solution seeped into the dialysate of membrane 2 but was still not visible in the dialysate of membrane 3 (Figure 3G-I). In fact, the Brilliant Blue solution was retained in the membrane up to the last timepoint, 72 hours (not pictured). After visual inspection of the membrane, no clear damage was observed. It may be that the dialysis pressure is too much for the clips to maintain the integrity of the closure, causing leakage of the solution into the dialysate. While folding the membrane prolonged retention of the solution within the membrane, it still leaked through after 8 hours. Therefore, membrane 3, the dialysis device, was deemed as the most appropriate MWCO and dialysis membrane to use.

Test 3 Figures:

Figure A.B.3. Brilliant blue dialysis testing

Figure A.B.3A-C Test 3 Brilliant Blue Dialysis – 6-hour timepoint

A) Membrane 1 B) Membrane 2 C) Membrane 3

Figure A.B.3D-F Test 3 Brilliant Blue Dialysis – 8-hour timepoint

D) Membrane 1 E) Membrane 2 F) Membrane 3

Figure A.B.3G-I Test 3 Brilliant Blue Dialysis – 24-hour timepoint

G) Membrane 1 H) Membrane 2 I) Membrane 3

Box 4. Do the dialysis devices retain phytic acid and allow phosphorus to freely dialyze?
Using the same sodium phytic acid and sodium phosphate solutions as described above, 5 mL of these solutions were pipet into the dialysis devices and dialyzed against 40 mL of ultrapure water for 4 hours. Dialysate was prepared in a 2.8% nitric acid solution and phosphorus was determined via MP-AES. Indeed, as shown in the bottom right figure, we found that the sodium phosphate solution reached equilibrium at 4 hours (99% bioaccessible phosphorus) and the percent of bioaccessible phosphorus from the sodium phytic acid solution was negligible (0.37%). However, The sodium phytic acid and sodium phosphate solutions contain different matricies than a food product. Therefore, we needed to determine the appropriate time to reach equilibrium dialysis when using food.

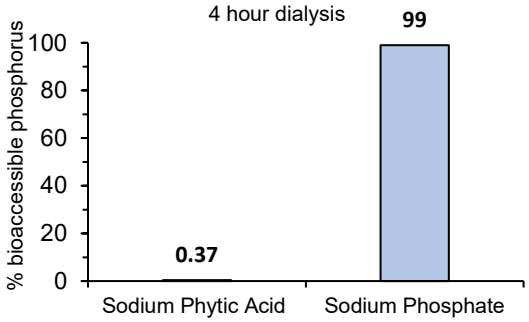


Figure A.B.4. Sodium phytic acid and sodium phosphate 4-hour dialysis test

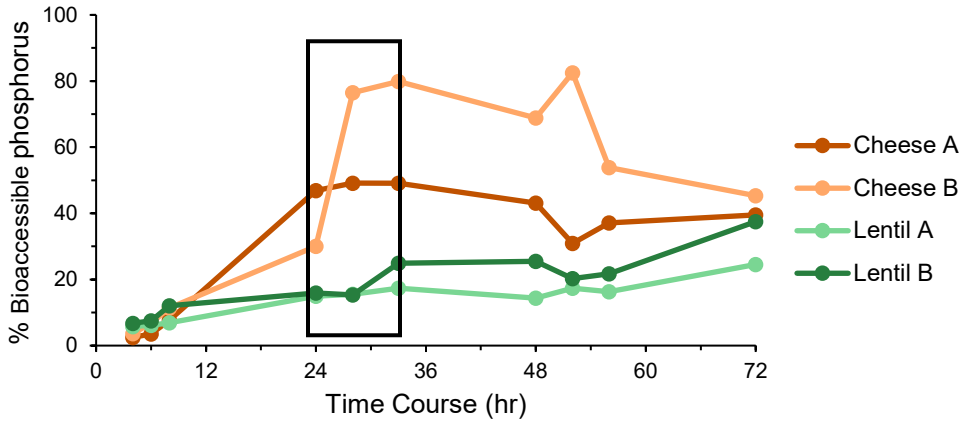
Test 4: Perform the *in vitro* digestion experiment on food products and subsequent dialysis to determine equilibrium dialysis.

Test 4 Methods:

1. 1g of freeze dried uncooked green lentils and cheese slices were put through the *in vitro* digestion experiments using the INFOGEST protocol.
2. Final digesta was diluted to 25 mL and was centrifuged at 4,000 rpm for 30 minutes to separate the soluble and insoluble fraction of the digesta.
3. 5 mL of the soluble fraction was pipet into the dialysis device and dialyzed against 40 mL of ultrapure water over the course of 72 hours at room temperature.
4. Prepare dialysate in 2.8% nitric acid solution to be analyzed for phosphorus content via MP-AES.

Test 4 Results: Over 72 hours of dialysis, equilibrium was determined to be reached around 30 hours for both lentils and cheese.

Figure A.B.5. Test 4 equilibrium dialysis



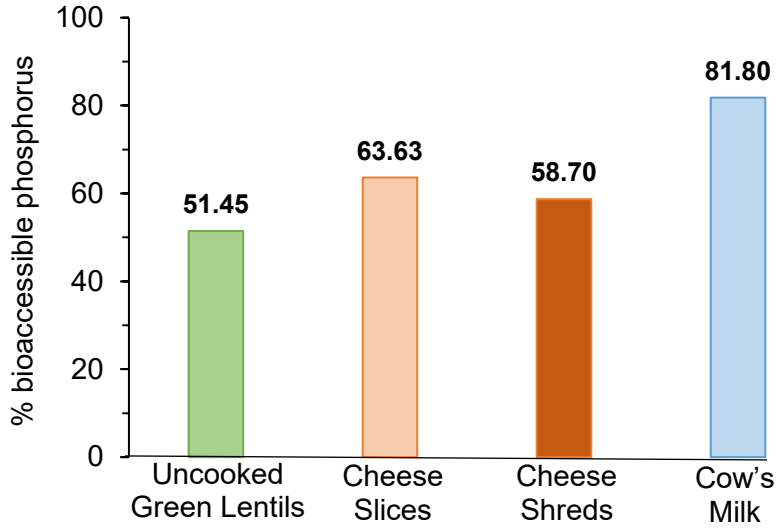
Test 5: Perform the *in vitro* digestion experiment on food products and subsequent dialysis using the 30-hour equilibrium dialysis determined in test 4.

Test 5 Methods:

1. 1g of freeze dried uncooked green lentils, cheese slices, cheese shreds, and cow's milk were put through the *in vitro* digestion experiments using the INFOGEST protocol.
2. Final digesta was diluted to 25 mL.
3. 5 mL of diluted digesta was pipetted into the dialysis device and dialyzed against 40 mL of ultrapure water for 30 hours at room temperature for determination of bioaccessible phosphorus.
    a. Upon completion of dialysis, dialysate was prepared in a 2.8% nitric acid solution.
4. 2-3g of diluted digesta was placed into porcelain crucibles and dry ashed in a muffle furnace for determination of total phosphorus content of digested samples.
    a. Samples were gradually heated to 60°C at a rate of 3°C/minute and held at 60°C for 24 hours.
    b. Samples were then heated to 600°C at a rate of 3°C/minute and held at 600°C for 24 hours
    c. Ashed samples were dissolved in 1 mL of concentrated nitric acid (70%) and diluted to 25 mL with ultrapure water.
5. Phosphorus content of the dialysate and ashed diluted digesta were determined via MP-AES

Test 5 Results: The percent bioaccessible phosphorus for the cheese products were ~59 and 64%. This is within the estimated range found in the literature for phosphorus from animal sources.[22] Phosphorus bioaccessibility for cow's milk was found to be 81.8%. This aligns with previously observed values for milk as Karp et al.[217] found 1.5% cow's milk to have 78.7% bioaccessible phosphorus. The percent bioaccessible phosphorus for uncooked green lentils was found to be ~51%. This is higher than what was observed by Karp et al.[216] but is similar to estimates of phosphorus bioavailability found by St. Jules et al.[136] There are many reasons why we may have found differences in phosphorus bioaccessibility in uncooked green lentils compared with Karp et al. including different methods were used for digestion and dialysis and that lentils were likely obtained from different suppliers/manufacturers and from different areas of the world.

Figure A.B.6. Test 5 in vitro digestion and subsequent dialysis 500-1000D MWCO



Conclusion: Throughout the method development process, we gained valuable insight on the most optimal way to perform the *in vitro* digestion experiment and subsequent dialysis to obtain accurate phosphorus bioaccessibility data. Based on these data, the dialysis device with a 500-1000D MWCO and dialysis time of 30 hours is required to determine phosphorus bioaccessibiltiy of food products.

Appendix C: Supplementary Information – Chapter 3: Phosphorus Bioaccessibilty of Emerging Pulse and Soy Protein Products by *In Vitro* Simulation of Human Digestion

Supplementary Methods

pH Testing

In preparation for the *in vitro* digestion experiments, all products underwent pH testing in which the amount of 5M HCl and 5M NaOH required to achieve a pH of 3 in the gastric phase and a pH of 7 in the intestinal phase was determined. Briefly, products were put through a test *in vitro* digestion experiment using the protocol described below except digestive enzymes were not utilized. After adding the required reagents to the gastric phase, the pH was tested and adjusted with known volumes of 5 M HCl or 5 M NaOH until reaching a pH of 3. This was repeated for the intestinal phase until a pH of 7 was reached. The amount of volume required to achieve the desired pH at each phase was recorded and utilized when performing the *in vitro* digestion experiments.

Bioaccessible Phosphorus Calculation

Ashed diluted digesta and dialysate of each sample were run on the MP-AES for determination of phosphorus on the same days with the same calibration curve and analytical blanks. Phosphorus of analytical blanks was averaged and subtracted from the experimental blank to accurately determine the contribution of background phosphorus from the *in vitro* digestion experiments. The contribution of background phosphorus was then subtracted from the phosphorus of the ashed diluted digesta samples to determine total phosphorus post-digestion. Contribution of background phosphorus was found to be negligible in the dialysate. The phosphorus values from the post-digestion and dialysate were used to calculate percent bioaccessible phosphorus. The percent bioaccessible phosphorus was then applied back to the total phosphorus of the whole food product to calculate the absolute bioaccesible phosphorus.

Table A.C.S1. Product details

Food Category	Protein Category	Product	Inorganic Phosphate Additives
Beef	Soy	Impossible - Plant-Based Burger Ground	N/A
		Incogmeato MorningStar Farms - Burger Patties	N/A
		Gardein - Plant-Based Ground Be'f	N/A
		MorningStar Farms - Vegan Meat Lovers (Patties)	N/A
	Pulse	Beyond Meat - Beyond Beef Beefy Crumbles	N/A
		Sweet Earth - Awesome Grounds	N/A
		Beyond Meat - Beyond Beef Plant Based Patties	N/A
		Gardein - Ultimate Plant-Based Burger	N/A
		Dr. Praeger's - All American Plant-Based Burgers	N/A
	Animal	Bubba Burger - Original Burger	N/A
		BallPark - Beef Patty	sodium phosphate
Milk	Soy	Silk - Original Soymilk	tricalcium phosphate
		Pacific Foods - Ultra Soy Plant-Based Beverage Original	tricalcium phosphate
		Silk - Ultra Original Soy Protein Beverage	N/A
	Pulse	Ripple - Unsweetened Plant-Based Milk Original	tricalcium phosphate; dipotassium phosphate
		Sproud - Plant-Based Milk Original	dipotassium phosphate
		Not Milk - Plant-Based Milk Alternative 2% Reduced Fat	dipotassium phosphate; monocalcium phosphate
	Animal	Kemps - 2% Reduced Fat Milk	N/A

Yogurt & Cheese	Soy	Silk - Dairy-Free Yogurt Vanilla	tricalcium phosphate; dipotassium phosphate
		Field Roast - Vegan Chao Shreds Creamy Original	N/A
		Field Roast - Vegan Chao Slices Creamy Original	N/A
	Pulse	Miyoko's - Cultured Vegan Cheese Farmhouse Cheddar	N/A
		Daiya - Cheddar Style Slices	N/A
	Animal	Yoplait - Original French Vanilla	N/A
		Kraft - Sharp Cheddar Shredded Cheese	N/A
		Kraft Singles - American	calcium phosphate; sodium phosphate
Sausage & Bacon	Soy	Gardein - Plant-Based Sliced Italian Saus'ge	N/A
		MorningStar Farms - Veggie Sausage Links	N/A
		MorningStar Farms - Veggie Bacon Strips	sodium phosphate; monocalcium phosphate; sodium tripolyphosphate
	Pulse	Beyond Meat - Beyond Sausage Plant-Based Links Brat Original	N/A
		Raised & Rooted - Bratwurst Plant Based Sausage	tetrasodium pyrophosphate
		Beyond Meat - Beyond Sausage Plant-Based Links Hot Italian	N/A
		Beyond Meat - Beyond Breakfast Sausage Plant-Based Links Classic	N/A
		Lightlife - Plant-Based Breakfast Patties	N/A
	Animal	Johnsonville - Original Bratwurst	N/A
		Johnsonville - Italian Sausage Mild	N/A
		Jimmy Dean - Original Pork Sausage Patties	sodium phosphate
		Hormel - Black Label Original Bacon	N/A

Chicken & Turkey	Soy	Wholesome Provisions - Just Like Chicken Textured Vegetable Protein	N/A
		MorningStar Farms - Original Chik Patties	sodium acid pyrophosphate
		Tofurky - Plant Based Deli Slices Oven Roasted	N/A
	Pulse	Raised & Rooted - Plant Based Nuggets	sodium acid pyrophosphate
		Beyond Meat - Beyond Chicken Plant Based Breaded Tenders	sodium acid pyrophosphate; monocalcium phosphate; sodium phosphates
	Animal	Tyson - Boneless Skinless Chicken Breasts	N/A
		Tyson - Chicken Patties	N/A
		Hillshire Farm - Oven Roasted Turkey Breast	sodium phosphate
Natural Forms & Traditional Processed Plant-Based Protein Products	Soy	Tofu	N/A
		LightLife Tempeh	N/A
		Bird's Eye Edamame	N/A
		Seapoint Farms Edamame Dry Roasted	N/A
		Soymerica Soybeans	N/A
	Pulses	Chickpeas (canned)	N/A
		Chickpeas (Dry)	N/A
		Green Lentils (Dry)	N/A
		Yellow Split Peas (Dry)	N/A
		Fava Bean (Canned)	N/A
		Fava Bean (Dry)	N/A
		Mung Bean (Dry)	N/A
	Peanut	Jif Peanut Butter (creamy)	N/A
	Soy	WowButter Soy Spread (creamy)	N/A
	Wheat	Barilla Pasta	N/A
	Soy	Simply Nature Soybean Spaghetti	N/A
	Pulse	Banza Spaghetti	N/A

Table A.C.S1. Product details.
Product details including food category, protein category, specific food product evaluated, and the presence of inorganic phosphate-containing additives found on the ingredient list for each product.

Table A.C.S2. Product cooking methods

Food Category	Protein Category	Product	Cooking Mode	Cooking Details
Beef	Soy	Impossible - Plant-Based Burger Ground	Skillet	Med-high heat; 8 min; from thawed
		Incogmeato MorningStar Farms - Burger Patties	Skillet	Med-high heat; 4 min per side; from frozen
		Gardein - Plant-Based Ground Be'f	Skillet	Med-high heat; 15 min; from frozen
		MorningStar Farms - Vegan Meat Lovers (Patties)	Skillet	Med-high heat; 18 min; from frozen
	Pulse	Beyond Meat - Beyond Beef Beefy Crumbles	Skillet	Med heat; 6 min; from frozen
		Sweet Earth - Awesome Grounds	Skillet	Med-high heat; 13 min; from frozen
		Beyond Meat - Beyond Beef Plant Based Patties	Skillet	Med-high heat; 10 min; from thawed
		Gardein - Ultimate Plant-Based Burger	Skillet	Med heat; 6-7 min per side; from frozen
		Dr. Praeger's - All American Plant-Based Burgers	Skillet	Med heat; 10 min per side; from frozen
	Animal	Bubba Burger - Original Burger	Skillet	Med heat; 7-10 min per side; from frozen
		BallPark - Beef Patty	Skillet	Med heat; 8-10 min; from frozen
Milk	Soy	Silk - Original Soymilk	N/A	
		Pacific Foods - Ultra Soy Plant-Based Beverage Original	N/A	
		Silk - Ultra Original Soy Protein Beverage	N/A	
	Pulse	Ripple - Unsweetened Plant-Based Milk Original	N/A	
		Sproud - Plant-Based Milk Original	N/A	
		Not Milk - Plant-Based Milk Alternative 2% Reduced Fat	N/A	
	Animal	Kemps - 2% Reduced Fat Milk	N/A	
Yogurt & Cheese	Soy	Silk - Dairy-Free Yogurt Vanilla	N/A	
		Field Roast - Vegan Chao Shreds Creamy Original	N/A	
		Field Roast - Vegan Chao Slices Creamy Original	N/A	
	Pulse	Miyoko's - Cultured Vegan Cheese Farmhouse Cheddar	N/A	

		Daiya - Cheddar Style Slices	N/A	
	Animal	Yoplait - Original French Vanilla	N/A	
		Kraft - Sharp Cheddar Shredded Cheese	N/A	
		Kraft Singles - American	N/A	
Sausage & Bacon	Soy	Gardein - Plant-Based Sliced Italian Saus'ge	Skillet	Med-high heat; 8 min
		MorningStar Farms - Veggie Sausage Links	Skillet	Med heat; 10 min
		MorningStar Farms - Veggie Bacon Strips	Skillet	Med heat; 10 min
	Pulse	Beyond Meat - Beyond Sausage Plant-Based Links Brat Original	Skillet	Med-high heat; 12 min
		Raised & Rooted - Bratwurst Plant Based Sausage	Skillet	Med heat; 10 min
		Beyond Meat - Beyond Sausage Plant-Based Links Hot Italian	Skillet	Med heat; 9 min
		Beyond Meat - Beyond Breakfast Sausage Plant-Based Links Classic	Skillet	Med heat; 10 min
		Lightlife - Plant-Based Breakfast Patties	Skillet	Med heat; 12 min
	Animal	Johnsonville - Original Bratwurst	Skillet	Med-high heat for 5 min; then added water and covered for 20 min
		Johnsonville - Italian Sausage Mild	Skillet	Med-high heat; 11 min
		Jimmy Dean - Original Pork Sausage Patties	Skillet	Med heat; 7 min
		Hormel - Black Label Original Bacon	Skillet	Med heat; 18-22 min
Chicken & Turkey	Soy	Wholesome Provisions - Just Like Chicken Textured Vegetable Protein	Skillet	Med-high heat; 7 min; 1:1 ratio of (chicken: water)
		MorningStar Farms - Original Chik Patties	Oven	375 degrees F; 16-18 min
		Tofurky - Plant Based Deli Slices Oven Roasted	N/A	
	Pulse	Raised & Rooted - Plant Based Nuggets	Oven	400 degrees F; 10 min
		Beyond Meat - Beyond Chicken Plant Based Breaded Tenders	Oven	425 degrees F; 4 min per side
	Animal	Tyson - Boneless Skinless Chicken Breasts	Oven	350 degrees F; 60 min
		Tyson - Chicken Patties	Oven	400 degrees F; 17-20 min

		Hillshire Farm - Oven Roasted Turkey Breast	N/A	
Natural Forms & Traditional Processed Plant-Based Protein Products	Soy	Tofu	Skillet	Med-high heat; 6 min (cubed)
		LightLife Tempeh	Skillet	Med-high heat; 8 min
		Bird's Eye Edamame	Microwave	Heat 3.5 min; let stand 1 min
		Seapoint Farms Edamame Dry Roasted	N/A	
		Soymerica Soybeans	Boil	Soaked in a 1:3 (bean:water) ratio overnight; Drained; Rinsed; Added to boiling water at same ratio for 3 hours
	Pulses	Chickpeas (canned)	Rinse	Rinsed with water
		Chickpeas (Dry)	Boil	Soaked in a 1:3 (pea:water) ratio overnight; Drained; Rinsed; Added to boiling water at same ratio for 1.5 hours
		Green Lentils (Dry)	Boil	Added to boiling water at 1:4 (lentil: water) ratio; simmered 20 min
		Yellow Split Peas (Dry)	Boil	Peas added to water in a 1:2 (pea:water) ratio; brought to boil; simmered 30 min
		Fava Bean (Canned)	Microwave	Drained but not rinsed; heated 3 min
		Fava Bean (Dry)	Boil	Soaked in a 1:10 (bean:water) ratio overnight; drained; peeled; boiled at same ratio for 10 min
		Mung Bean (Dry)	Boil	Soaked in a 1:3 (bean:water) ratio for 15 min; brought to boil in same ratio; simmered 30 min; let stand 10 min
	Peanut	Jif Peanut Butter (creamy)	N/A	
	Soy	WowButter Soy Spread (creamy)	N/A	
	Wheat	Barilla Pasta	Boil	10 min
	Soy	Simply Nature Soybean Spaghetti	Boil	4.5 min; 8 cups water used to boil entire package
	Pulse	Banza Spaghetti	Boil	9 min

Table A.C.S2. Product cooking methods.
Cooking mode and details for each evaluated product.

Table A.C.S3. Phosphorus bioaccessibility

Food Category	Protein Category	Product	Total P (mg/100g)	Absolute Bioaccessible P (mg/100g)	Percent Bioaccessible P (%)	Number of Inorganic Phosphate Additives
Beef	Soy	Impossible - Plant-Based Burger Ground	167	102	61	0
		Incogmeato MorningStar Farms - Burger Patties	212	77	36	0
		Gardein - Plant-Based Ground Be'f	198	100	51	0
		MorningStar Farms - Vegan Meat Lovers (Patties)	140	82	59	0
	Pulse	Beyond Meat - Beyond Beef Beefy Crumbles	241	56	23	0
		Sweet Earth - Awesome Grounds	284	74	26	0
		Beyond Meat - Beyond Beef Plant Based Patties	273	98	36	0
		Gardein - Ultimate Plant-Based Burger	239	101	42	0
		Dr. Praeger's - All American Plant-Based Burgers	283	89	31	0
	Animal	Bubba Burger - Original Burger	169	168	99	0

		BallPark - Beef Patty	161	127	79	1
Milk	Soy	Silk - Original Soymilk	91	41	45	1
		Pacific Foods - Ultra Soy Plant-Based Beverage Original	97	57	58	1
		Silk - Ultra Original Soy Protein Beverage	105	57	54	0
	Pulse	Ripple - Unsweetened Plant-Based Milk Original	132	114	86	2
		Sproud - Plant-Based Milk Original	143	169	118	1
		Not Milk - Plant-Based Milk Alternative 2% Reduced Fat	157	166	106	2
	Animal	Kemps - 2% Reduced Fat Milk	75	63	85	0
Yogurt & Cheese	Soy	Silk - Dairy-Free Yogurt Vanilla	125	93	74	2
		Field Roast - Vegan Chao Shreds Creamy Original	5	0	0	0
		Field Roast - Vegan Chao	7	0	0	0

		Slices Creamy Original				
	Pulse	Miyoko's - Cultured Vegan Cheese Farmhouse Cheddar	141	90	64	0
		Daiya - Cheddar Style Slices	345	214	62	0
	Animal	Yoplait - Original French Vanilla	105	86	81	0
		Kraft - Sharp Cheddar Shredded Cheese	402	359	89	0
		Kraft Singles - American	1223	1547	93	2
Sausage & Bacon	Soy	Gardein - Plant-Based Sliced Italian Saus'ge	207	123	59	0
		MorningStar Farms - Veggie Sausage Links	80	77	96	0
		MorningStar Farms - Veggie Bacon Strips	293	316	108	3
	Pulse	Beyond Meat - Beyond Sausage Plant-Based Links Brat Original	264	124	47	0
		Raised & Rooted -	196	118	60	1

		Bratwurst Plant Based Sausage				
		Beyond Meat - Beyond Sausage Plant-Based Links Hot Italian	186	132	71	0
		Beyond Meat - Beyond Breakfast Sausage Plant-Based Links Classic	267	123	46	0
		Lightlife - Plant-Based Breakfast Patties	186	116	62	0
	Animal	Johnsonville - Original Bratwurst	122	132	108	0
		Johnsonville - Italian Sausage Mild	143	133	93	0
		Jimmy Dean - Original Pork Sausage Patties	173	177	103	1
		Hormel - Black Label Original Bacon	237	262	111	0
Chicken & Turkey	Soy	Wholesome Provisions - Just Like Chicken Textured Vegetable Protein	196	97	49	0

		MorningStar Farms - Original Chik Patties	184	158	86	1
		Tofurky - Plant Based Deli Slices Oven Roasted	146	151	104	0
	Pulse	Raised & Rooted - Plant Based Nuggets	156	118	76	1
		Beyond Meat - Beyond Chicken Plant Based Breaded Tenders	116	93	80	3
	Animal	Tyson - Boneless Skinless Chicken Breasts	260	278	107	0
		Tyson - Chicken Patties	125	156	125	0
		Hillshire Farm - Oven Roasted Turkey Breast	273	270	99	1
Natural Forms & Traditional Processed Plant-Based	Soy	Tofu	145	69	48	0
		LightLife Tempeh	260	151	58	0
		Bird's Eye Edamame	172	109	63	0
		Seapoint Farms Edamame Dry Roasted	571	348	61	0

Protein Products		Soymerica Soybeans	186	94	51	0
	Pulses	Chickpeas (canned)	52	53	102	0
		Chickpeas (Dry)	104	71	68	0
		Green Lentils (Dry)	128	116	91	0
		Yellow Split Peas (Dry)	130	98	76	0
		Fava Bean (Canned)	73	75	103	0
		Fava Bean (Dry)	166	101	61	0
		Mung Bean (Dry)	121	95	78	0
	Peanut	Jif Peanut Butter (creamy)	324	226	70	0
	Soy	WowButter Soy Spread (creamy)	349	309	88	0
	Wheat	Barilla Pasta	65	62	95	0
	Soy	Simply Nature Soybean Spaghetti	204	97	47	0
	Pulse	Banza Spaghetti	100	77	77	0

Table A.C.S3. Phosphorus bioaccessibility.
Total, absolute bioaccessible, percent bioaccessible phosphorus and number of inorganic phosphate containing additives identified on the ingredient list for each food product evaluated

Appendix D: Supplementary Information – Chapter 4: Effects of Plant-Based Protein Consumption on Kidney Function and Mineral Bone Disorder Outcomes in Adults with Stage 3-5 Chronic Kidney Disease: A Systematic Review

Table A.D.S1. Query strings for databases searched

A. Medline Search Strategy

Step	Search
1	exp renal insufficiency, chronic/
2	chronic renal insufficienc*.mp.
3	chronic kidney disease*.mp.
4	chronic renal disease*.mp.
5	CKD.mp.
6	chronic renal failure.mp.
7	advance* renal failure.mp.
8	advance* kidney failure.mp.
9	((kidney or renal) adj2 (disease* or failure* or end-stage*)) ti,ab.
10	9 and chronic.ti,ab.
11	or/1-10
12	exp plant proteins, dietary/
13	exp vegetarians/
14	exp vegans/
15	exp dietary proteins/
16	dietary plant protein*.mp.
17	plant-based nutrition.mp.
18	plant-based diet*.mp.
19	plant diet*.mp.
20	vegetable protein*.mp.
21	vegetable product*.mp.
22	plant product*.mp.
23	plant protein product*.mp.
24	plant food*.mp.
25	protein from plant*.mp.
26	meat replace*.mp.
27	meatless*.mp.
28	meat alternative*.mp.
29	meat substitute*.mp.
30	plant based meat*.mp.
31	meat analog*.mp.
32	vegetarian*.mp.

33	vegan*.mp.
34	lacto-ovo vegetarian*.mp.
35	ovo-lacto vegetarian*.mp.
36	lacto vegetarian*.mp.
37	ovo vegetarian*.mp.
38	lacto*veg*.mp.
39	ovo*veg*.mp.
40	lacto*ovo.mp.
41	ovo*lacto*.mp.
42	((lacto or ovo) adj2 veg*).ti,ab.
43	semi*vegetarian*.mp.
44	flexitarian*.mp.
45	pescatarian*.mp.
46	pesco*vegetarian*.mp.
47	nordic diet.mp.
48	daniel fast*.mp.
49	mediterranean diet.mp.
50	paleo*diet.mp.
51	DASH diet.mp.
52	ornish diet.mp.
53	MIND diet.mp.
54	low gi diet.mp.
55	prudent diet.mp.
56	portfolio diet.mp.
57	protein intake*.mp.
58	protein consum*.mp.
59	protein absor*.mp.
60	protein supplement*.mp.
61	or/12-60
62	11 and 61
63	62 nd english.lg

B. Embase Search Strategy

Step	Search
1	exp kidney failure/
2	chronic renal insufficienc*.mp.
3	chronic kidney disease*.mp.
4	chronic renal disease*.mp.
5	CKD.mp.
6	chronic renal failure.mp.
7	advance* renal failure.mp.
8	advance* kidney failure.mp.

9	((kidney or renal) adj2 (disease* or failure* or end-stage*)) ti,ab.
10	9 and chronic.ti,ab.
11	or/1-10
12	exp plant protein/
13	exp vegetarian/
14	exp vegan/
15	exp vegan diet/
16	exp protein intake/
17	dietary plant protein*.mp.
18	plant-based nutrition.mp.
19	plant-based diet*.mp.
20	plant diet*.mp.
21	vegetable protein*.mp.
22	vegetable product*.mp.
23	plant product*.mp.
24	plant protein product*.mp.
25	plant food*.mp.
26	protein from plant*.mp.
27	meat replace*.mp.
28	meatless*.mp.
29	meat alternative*.mp.
30	meat substitute*.mp.
31	plant based meat*.mp.
32	meat analog*.mp.
33	vegetarian*.mp.
34	vegan*.mp.
35	lacto-ovo vegetarian*.mp.
36	ovo-lacto vegetarian*.mp.
37	lacto vegetarian*.mp.
38	ovo vegetarian*.mp.
39	lacto*veg*.mp.
40	ovo*veg*.mp.
41	((lacto or ovo) adj2 veg*).ti,ab.
42	semi*vegetarian*.mp.
43	flexitarian*.mp.
44	pescatarian*.mp.
45	pesco*vegetarian*.mp.
46	nordic diet.mp.
47	daniel fast*.mp.
48	mediterranean diet.mp.
49	paleo*diet.mp.
50	DASH diet.mp.

51	ornish diet.mp.
52	MIND diet.mp.
53	low gi diet.mp.
54	prudent diet.mp.
55	portfolio diet.mp.
56	protein intake*.mp.
57	protein consum*.mp.
58	protein absor*.mp.
59	protein supplement*.mp.
60	or/12-59
61	11 and 60
62	61 and english.lg

C. CAB Abstracts Search Strategy

Step	Search
1	chronic renal insufficienc*.mp.
2	chronic kidney disease*.mp.
3	chronic renal disease*.mp.
4	CKD.mp.
5	chronic renal failure.mp.
6	advance* renal failure.mp.
7	advance* kidney failure.mp.
8	((kidney or renal) adj2 (disease* or failure* or end-stage*)) ti,ab.
9	8 and chronic.ti,ab.
10	or/1-9
11	dietary plant protein*.mp.
12	plant-based nutrition.mp.
13	plant-based diet.mp.
14	plant diet*.mp.
15	vegetable protein*.mp.
16	vegetable product*.mp.
17	plant product*.mp.
18	plant protein product*.mp.
19	plant food*.mp.
20	protein from plant*.mp.
21	meat replace*.mp.
22	meatless*.mp.
23	meat alternative*.mp.
24	meat substitute*.mp.
25	plant based meat*.mp.
26	meat analog*.mp.
27	vegetarian*.mp.

28	vegan*.mp.
29	lacto-ovo vegetarian*.mp.
30	ovo-lacto vegetarian*.mp.
31	lacto vegetarian*.mp.
32	ovo vegetarian*.mp.
33	lacto*veg*.mp.
34	ovo*veg*.mp.
35	ovo*lacto*.mp.
36	lacto*ovo*.mp.
37	((lacto or ovo) adj2 veg*).ti,ab.
38	semi*vegetarian*.mp.
39	flexitarian*.mp.
40	pescatarian*.mp.
41	pesco*vegetarian*.mp.
42	nordic diet.mp.
43	daniel fast*.mp.
44	mediterranean diet.mp.
45	paleo*diet.mp.
46	DASH diet.mp.
47	ornish diet.mp.
48	MIND diet.mp.
49	low gi diet.mp.
50	prudent diet.mp.
51	portfolio diet.mp.
52	protein intake*.mp.
53	protein consum*.mp.
54	protein absor*.mp.
55	protein supplement*.mp.
56	or/11-55
57	10 and 56
58	57 and english.lg

D. Agricola Search Strategy

Step	Search
1	chronic renal insufficiency.mp.
2	chronic kidney disease*.mp.
3	chronic renal disease*.mp.
4	CKD.mp.
5	chronic renal failure.mp.
6	advance* renal failure.mp.
7	advance* kidney failure.mp.
8	((kidney or renal) adj2 (disease* or failure* or end-stage*)) ti,ab.

9	**8 and chronic.ti,ab.**
10	**or/1-9**
11	**dietary plant protein*.mp.**
12	**plant-based nutrition.mp.**
13	**plant-based diet.mp.**
14	**plant diet*.mp.**
15	**Vegetable protein*.mp.**
16	**Vegetable product*.mp.**
17	**Plant product*.mp.**
18	**Plant protein product*.mp.**
19	**Plant food*.mp.**
20	**Protein from plant*.mp.**
21	**Meat replace*.mp.**
22	**meatless*.mp.**
23	**Meat alternative*.mp.**
24	**Meat substitute*.mp.**
25	**Plant based meat*.mp.**
26	**Meat analog*.mp.**
27	**vegetarian*.mp.**
28	**vegan*.mp.**
29	**Lacto-ovo vegetarian*.mp.**
30	**Ovo-lacto vegetarian*.mp.**
31	**Lacto vegetarian*.mp.**
32	**Ovo vegetarian*.mp.**
33	**Lacto*veg*.mp.**
34	**Ovo*veg*.mp.**
35	**Ovo*lacto*.mp.**
36	**Lacto*ovo*.mp.**
37	**((lacto or ovo) adj2 veg*).ti,ab.**
38	**Semi*vegetarian*.mp.**
39	**flexitarian*.mp.**
40	**pescatarian*.mp.**
41	**Pesco*vegetarian*.mp.**
42	**Nordic diet.mp.**
43	**Daniel fast*.mp.**
44	**Mediterranean diet.mp.**
45	**Paleo*diet.mp.**
46	**DASH diet.mp.**
47	**Ornish diet.mp.**
48	**MIND diet.mp.**
49	**Low GI diet.mp.**
50	**Prudent diet.mp.**

51	**Portfolio diet.mp.**
52	**Protein intake*.mp.**
53	**Protein consum*.mp.**
54	**Protein absor*.mp.**
55	**Protein supplement*.mp.**
56	**Or/11-55**
57	**10 and 56**
58	**57 and English.lg**

E. Web of Science Search Strategy
Query: #1 AND #2 AND #3

Query #1: (((((((((TS=("chronic renal insufficienc*")) OR TS=("chronic kidney disease*")) OR TS=("chronic renal disease*")) OR TS=("CKD")) OR TS=("chronic renal failure")) OR TS=("advance* renal failure")) OR TS=("advance* kidney failure")) OR TS=("end stage renal disease")) OR TS=("end stage kidney disease"))

Query #2: ((TS=("dietary plant protein*")) OR TS=("plant based nutrition")) OR TS=("plant-based nutrition")) OR TS=("plant-based diet")) OR TS=("plant based diet")) OR TS=("plant diet*")) OR TS=("vegetable protein*")) OR TS=("vegetable product*")) OR TS=("plant product*")) OR TS=("plant protein product*")) OR TS=("plant food*")) OR TS=("protein from plant*")) OR TS=("meat replace*")) OR TS=("meatless*")) OR TS=("meat alternative*")) OR TS=("meat substitute*")) OR TS=("plant based meat*")) OR TS=("meat analog*")) OR TS=("vegetarian*")) OR TS=("vegan*")) OR TS=("lacto-ovo vegetarian*")) OR TS=("ovo-lacto vegetarian*")) OR TS=("lacto vegetarian*")) OR TS=("ovo vegetarian*")) OR TS=("lacto*veg*")) OR TS=("ovo*veg*")) OR TS=("ovo*lacto*")) OR TS=("lacto*ovo*")) OR TS=("semi*vegetarian*")) OR TS=("flexitarian*")) OR TS=("pescatarian*")) OR TS=("pesco*vegetarian*")) OR TS=("Nordic diet")) OR TS=("daniel fast")) OR TS=("Mediterranean diet")) OR TS=("Paleo* diet")) OR TS=("DASH diet")) OR TS=("Ornish diet")) OR TS=("MIND diet")) OR TS=("low GI diet")) OR TS=("Prudent diet")) OR TS=("Portfolio diet")) OR TS=("protein intake*")) OR TS=("protein consum*")) OR TS=("protein absor*")) OR TS=("protein supplement*"))

Query #3: LA=(English)

F. Scopus Search Strategy

TITLE-ABS-KEY ("chronic renal insufficienc*" OR "chronic kidney disease*" OR "chronic renal disease*" OR "CKD" OR "chronic renal failure" OR "advance* renal failure" OR "advance* kidney failure" OR "end stage renal disease" OR "end stage kidney disease") AND TITLE-ABS-KEY ("dietary plant protein*" OR "plant based nutrition" OR "plant-based nutrition" OR "plant-based diet" OR "plant based diet" OR "plant diet*" OR "vegetable protein*" OR "vegetable product*" OR "plant product*" OR "plant protein product*" OR "plant food*" OR "protein from plant*" OR "meat replace*" OR "meatless*" OR "meat alternative*" OR "meat substitute*" OR "plant based meat*" OR "meat analog*" OR "vegetarian*" OR "vegan*" OR "lacto-ovo vegetarian*" OR "ovo-lacto vegetarian*" OR "lacto vegetarian*" OR "ovo vegetarian*" OR "lacto*veg*" OR "ovo*veg*" OR "ovo*lacto*" OR "lacto*ovo*" OR "semi*vegetarian*" OR "flexitarian*" OR "pescatarian*" OR "pesco*vegetarian*" OR "Nordic diet" OR "daniel fast" OR "Mediterranean diet" OR "Paleo* diet" OR "DASH diet" OR "Ornish diet" OR "MIND diet" OR "low GI diet" OR "Prudent diet" OR "Portfolio diet" OR "protein intake*" OR "protein consum*" OR "protein absor*" OR "protein supplement*") AND LANGUAGE (english)

Supplementary Table 1 Query strings for databases searched. MeSH terms and keywords were incorporated based on the specifications for each database. **A)** Medline (1966-July 2021) **B)** Embase (1947-July 2021) **C)** Agricola (1970-July 2021) **D)** CAB Abstracts (1973-July 2021) **E)** Web of Science (1900-July 2021) **F)** Scopus (1966-July 2021)

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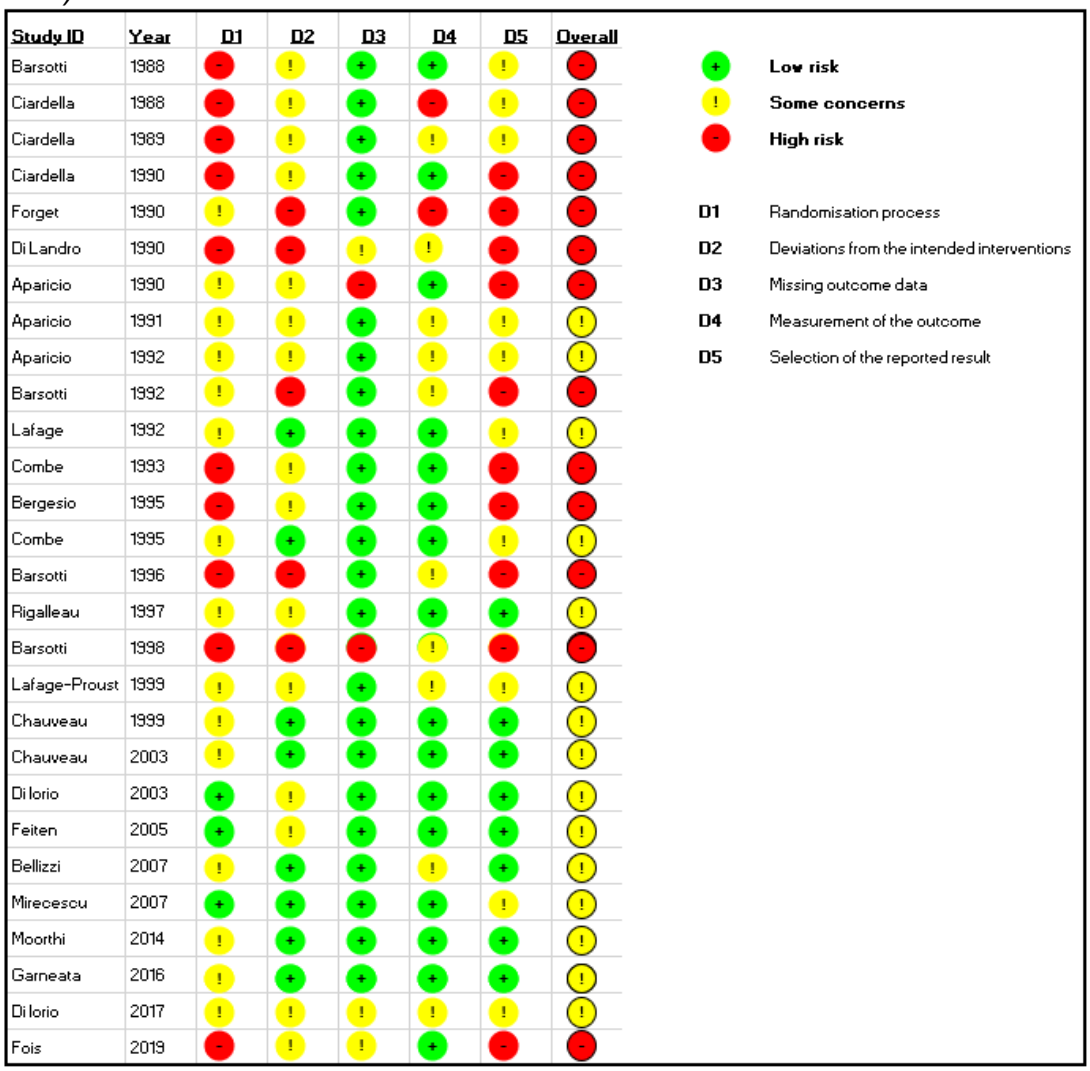
Figure A.D.S1. Risk of bias assessment summary by domain for all included studies

A) RoB2 for RCTs



B) RoB2 for Crossover Trials

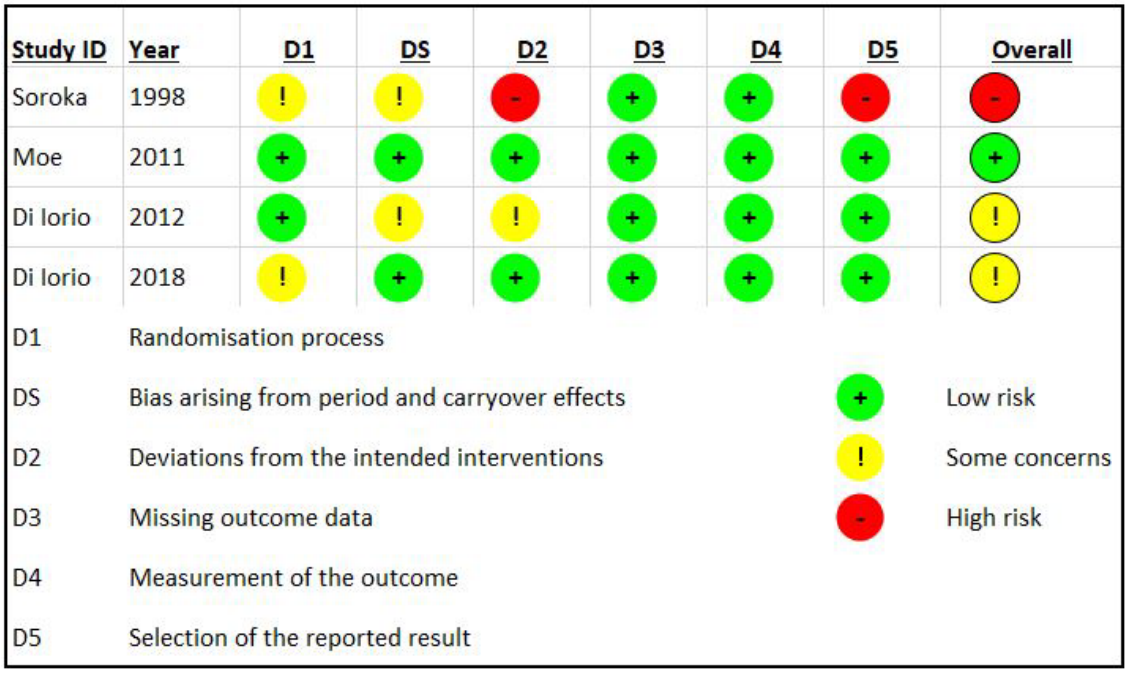


Figure A.D.S1. A) Summary of domain assessment for RCTs. Overall, 15 of studies were considered moderate risk of bias while 13 were considered high risk of bias. **B) Summary of domain assessment for crossover trials.** Overall, 1 study was considered low, 2 moderate, and 1 high risk of bias. Domain categories are listed to the side or below each table. A green circle with a plus sign indicates low risk of bias, a yellow circle with an exclamation point indicates moderate risk of bias, and a red circle with a minus sign indicates high risk of bias.

Supplementary Summary of all included 32 studies:

Kidney function outcomes:

For CrCl or eGFR, 11 short and mid-length duration studies.[305–311,313,314,316,318] observed no change. One mid-length study[312] with a small sample size (n=21), high risk of bias and no control group showed a decrease in CrCl after a very low protein vegetarian diet. However, studies of longer duration observed mixed results, with 8 studies[319,321–325,333,335] reporting no change and 6 studies[326–329,331,332] reporting a decrease in CrCl or eGFR following the diet intervention. For BUN or urea, regardless of study duration, 23 of 25 studies observed a decrease with a medium or large magnitude of effect (when effect size could be determined).

Renal replacement therapy (RRT) initiation was reported in 6 of the 32 studies. Of the mid-length studies, Fois et al.[308] and Di Iorio et al.[309] did not report significance for the number of subjects who initiated RRT based on intervention. Bellizzi et al.[310] observed no RRT initiation regardless of diet intervention. Of the long-term studies, an earlier study[328] found no significant difference in RRT initiation between participants who were compliant versus non-compliant with a very low protein vegetarian diet. However, more recently, Mircescu et al.[321] and Garneata et al.,[320] which were a pilot study and subsequent full trial from the same group, observed fewer participants with RRT initiation or reduced eGFR >50% on the very low protein vegetarian diet compared with a traditional low protein diet.

CKD-MBD Outcomes:

For serum phosphorus, regardless of study duration, mixed results were observed for effect of the interventions with 7 studies[305,311,313,314,322,326,334] reporting no change and 14 studies[306,307,309,310,312,319,321,323,325,327,328,330,331,335] reporting a decrease. For serum PTH, studies of short duration found no change,[305–307] studies of mid-length found either no change[308,311,314] or a decrease,[309,310,312,315,316,318] and all studies of long duration found a decrease.[319,322–325,327,328,330–336] Cardiovascular events were not reported in any study included in this systematic review. Bone outcomes were reported in 5 of the 32 included studies.[322,324,325,329,331] Briefly, following 12 months of a very low protein vegetarian diet, Lafage et al.[331] observed reduced osteomalacia and osteitis fibrosa, increased mineral apposition rate and bone formation rate, and decreased osteoid thickness in 4 participants with mixed osteopathy. Further, in 9 participants with osteitis fibrosa, 5 had improvements in the disorder while 4 had no improvements. However, after 65 ± 24 months of a very low protein vegetarian diet, Lafage-Proust et al.[325] observed severe or moderate bone loss at the femoral neck (N=12), at the radius (N=8), and at the spine (N=2).

Table A.D.S2. Summary of kidney function outcomes all studies

Study	Study Duration Category	Duration	N	Treatment	Direction of change			
					CrCl or eGFR (mL/min/1.73 m^2)	Serum Creatinine (mg/dL)	Serum BUN or Urea (mg/dL)	Proteinuria (g/24 hr)
Moorthi 2014[305]	Short term (1-4 weeks)	4 wk	13	70% plant prt E vs B	⟷	↓	NR	NR
Di Iorio 2012[306]		1 wk	32	VLPD vs LPD	⟷	⟷	↓↓	NR
Moe 2011[307]		1 wk/diet	8	Veg. vs Meat	⟷	NR	NR	NR
Fois 2019[308]	Mid-length (>4-26 weeks)	≥ 3mo	65	LPD veg. vs LPD mix	⇔	⟷	⇔	⇔
Di Iorio 2018[309]		6 mo	60	VLPD vs MD	⟷	⟷	↓↓↓	↓
Bellizzi 2007[310]		6 mo	110	VLPD vs LPD	⟷	NR	↓↓↓	⟷
Feiten 2005[311]		4 mo	24	VLPD vs LPD	⟷	⟷	↓↓	NR
Barsotti 1998[312]		4 ± 2 mo	21	VLPD E vs B	↓↓	⟷	↓↓↓	NR
Soroka 1998[313]		6 mo	9	Veg. vs Mixed	⟷ ⟷ ⟷	⟷	↓↓↓	⟷
Rigallea u 1997[314]		3 mo	8	VLPD E vs B	⟷	⟷	↓↓↓	NR
Bergesio 1995[315]		≥ 3mo	21	VLPD vs LPD	NR	⟷	NR	NR
Ciardella 1990[316]		4-8 mo	13	VLPD E vs B	⟷	NR	NR	NR
Forget 1990[a][317]		6 mo	32	VLPD vs LPD	NR	NR	NR	NR
Ciardella 1989[318]		1-4 mo	12	VLPD E vs B	⟷	NR	NR	NR
Di Iorio 2017[319]	Long term (>26 weeks)	12 mo	146	VLPD vs LPD	⟷	⟷	↓↓↓	↓↓
Garneata 2016[320]		15 mo	159	VLPD vs LPD	[b]⇩	NR	⇩	⇔
Mircescu 2007[321]		48 wks	45	VLPD E vs B	⇄ ⇄	↑↑	↓↓↓	⟷ ⇄ ⇄

Chauveau 2003[322]		24 mo	13	VLPD E vs B	⇄	↔	↓↓↓	↔
Di Iorio 2003[323]		18 mo	20	VLPD vs LPD	↔	**NR**	↓↓↓	**NR**
Chauveau 1999[324]		12 mo	10	VLPD E vs B	↔	↔	↓↓↓	↓↓
Lafage-Proust 1999[325]		65 ± 24.4 mo	16	VLPD HBFR vs NBFR vs LBFR	⇔	**NR**	**NR**	**NR**
Barsotti 1996[326]		12.8 ± 5.7 mo	11	LPD, mixed to LPD Veg.	⇩	⇔	⇔	**NR**
		14.1 ± 5.0 mo	11	FD to LPD Veg.	⇩	⇔	⇩	**NR**
Combe 1995[327]		24 mo	29	VLPD E vs B	↓↓	↔	↓↓↓	**NR**
Combe 1993[328]		23.0 ± 10.6 mo 23.7 ± 11.6 mo	40	VLPD-C vs VLPD-NC	⇩	**NR**	↓↓↓	↓↓↓
Aparicio 1992[329]		19.2 ± 7.6 mo 19.9 ± 8.1 mo	40	VLPD-C vs VLPD-NC	↓	↔	↓↓↓	**NR**
Barsotti 1992[330]		8-58 mo	23	VLPD or LPD Veg. E vs B	**NR**	**NR**	**NR**	↓↓↓
Lafage 1992[331]		12 mo	17	VLPD E vs B	↓	↔	↓↓↓	**NR**
Aparicio 1991[332]		12 mo	20	VLPD E vs B	↓	↔	↓↓↓	**NR**
Aparicio 1990[333]		≥18 mo	66	VLPD-C E vs B	↔	↔	↓↓↓	↓
Di Landro 1990[334]		36 mo	69	VLPD vs LPD	**NR**	⇩	⇩	**NR**
Ciardella 1988[335]		13.6 ± 6.9 mo	11	VLPD E vs B	⇔	**NR**	⇩	⇩
Barsotti 1988[336]		17.4 ± 5.8 mo	8	VLPD or LPD Veg. E vs B	**NR**	**NR**	↓↓↓	↓↓↓

Table A.D.S2. Summary of kidney function outcomes all studies
Direction of change and effect sizes for CrCl or eGFR, serum creatinine and BUN, and proteinuria is shown for all studies. For all outcomes mixed results were observed across studies. Horizontal arrows indicate no change, up arrows indicate an increase, and down arrows indicate a decrease in the outcome reported. Effect size is shown by arrow number with one arrow representing a small magnitude of effect, two arrows representing a medium magnitude of effect, and three arrows representing a large magnitude of effect. White arrows were used when effect size was unable to be determined. *NR* not reported, *prt* protein, *E* endpoint, *B* baseline, *LPD* low protein diet, *veg* vegetarian *MD* Mediterranean diet, *HBFR* high bone formation rate, *NBFR* normal bone formation rate, *LBFR* low bone formation rate, *FD* free diet, *VLPD-C* very low protein diet compliant, *VLPD-NC* very low protein diet non-compliant [a]No statistical tests were performed. Thus, statistical differences in outcomes were not reported for this study and true direction of changes unable to be determined. [b]Decreased on LPD compared with VLPD. All reported values for outcomes can be found in **Supplementary File 3** (https://www.doi.org/10.17605/OSF.IO/YFR57).

Table A.D.S3. Summary of CKD-MBD outcomes all studies

Study	Study Duration Category	Duration	N	Treatment	Direction of change			
					Serum P (mg/dL)	Serum PTH (pg/mL)	Serum Ca (mg/dL)	Urinary P (mg/24 hr)
Moorthi 2014[305]	Short term (1-4 weeks)	4 wks	13	70% plant prt E vs B	↔	↔	↔	↓↓↓
Di Iorio 2012[306]		1 wk	32	VLPD vs LPD	↓↓↓	↔	↔	↓↓↓
Moe 2011[307]		1 wk/diet	8	Veg. vs meat	↓↓↓	[b] ↓	⇅	⇅
Fois 2019[308]	Mid-length (>4-26 weeks)	≥3 mo	65	LPD veg. vs LPD mix	**NR**	⇔	**NR**	**NR**
Di Iorio 2018[309]		6 mo	60	VLPD vs MD	↓↓↓	↓	↔	↓↓↓
Bellizzi 2007[310]		6 mo	110	VLPD vs LPD	↓↓↓	↓↓	**NR**	↓↓↓
Feiten 2005[311]		4 mo	24	VLPD vs LPD	↔	↔	↔	↓↓↓
Barsotti 1998[312]		4 ± 2 mo	21	VLPD E vs B	↓↓↓	↓↓↓	↑↑↑	**NR**
Soroka 1998[313]		6 mo	9	Veg. vs mix	⇅	**NR**	⇅	↓↓↓
Rigalleau 1997[314]		3 mo	8	VLPD E vs B	↔	↔	↔	**NR**
Bergesio 1995[315]		≥ 3mo	21	VLPD vs LPD	**NR**	↓↓↓	**NR**	**NR**
Ciardella 1990[316]		4-8 mo	13	VLPD E vs B	**NR**	↓↓↓	**NR**	**NR**
Forget 1990[a317]		6 mo	32	VLPD vs LPD	**NR**	**NR**	**NR**	**NR**
Ciardella 1989[318]		1-4 mo	12	VLPD E vs B	**NR**	↓↓	**NR**	**NR**
Di Iorio 2017[319]	Long term (>26 weeks)	12 mo	146	VLPD vs LPD	↓↓↓	↓↓↓	↓↓	[c] ↓
Garneata 2016[320]		15 mo	159	VLPD vs LPD	⇩	**NR**	⇧	**NR**
Mircescu 2007[321]		48 wks	45	VLPD E vs B	↓↓	**NR**	↑↑	**NR**
Chauveau 2003[322]		24 mo	13	VLPD E vs B	↔	↓↓↓	⇅	**NR**

Di Iorio 2003[323]		18 mo	20	VLPD vs LPD	⬇⬇⬇	⬇⬇⬇	⇔	⬇⬇⬇
Chauveau 1999[324]		12 mo	10	VLPD E vs B	**NR**	⬇⬇⬇	**NR**	**NR**
Lafage-Proust 1999[325]		65 ± 24.4 mo	16	VLPD HBFR vs NBFR vs LBFR	[d]⇩	[e]⇩	⇔	⇩
Barsotti 1996[326]		12.8 ± 5.7 mo	11	LPD mixed to LPD veg.	⇔	**NR**	**NR**	**NR**
		14.1 ± 5.0 mo	11	FD to LPD Veg.	⇔	**NR**	**NR**	**NR**
Combe 1995[327]		24 mo	29	VLPD E vs B	⬇⬇⬇	⬇⬇	⬆⬆	⬇⬇⬇
Combe 1993[328]		23.0 ± 10.6 mo 23.7 ± 11.6 mo	40	VLPD-C vs VLPD-NC	⬇⬇⬇	⬇⬇⬇	⬌	**NR**
Aparicio 1992[329]		19.2 ± 7.6 mo 19.9 ± 8.1 mo	40	VLPD-C vs VLPD-NC	--	--	--	--
Barsotti 1992[330]		8-58 mo	23	VLPD or LPD Veg. E vs B	⬇⬇⬇	⬇⬇⬇	⬆⬆⬆	**NR**
Lafage 1992[331]		12 mo	17	VLPD E vs B	⬇⬇	⬇⬇⬇	⬌	⬇⬇⬇
Aparicio 1991[332]		12 mo	20	VLPD E vs B	**NR**	⬇⬇⬇	**NR**	**NR**
Aparicio 1990[333]		≥18 mo	66	VLPD-C E vs B	⬇	⬇⬇⬇	⬆	⬇
Di Landro 1990[334]		36 mo	69	VLPD vs LPD	⇔	⇩	⇔	**NR**
Ciardella 1988[335]		13.6 ± 6.9 mo	11	VLPD E vs B	**NR**	⬇⬇⬇	**NR**	**NR**
Barsotti 1988[336]		17.4 ± 5.8 mo	8	VLPD or LPD Veg. E vs B	**NR**	⬇	**NR**	**NR**

Table A.D.S3. Summary of CKD-MBD outcomes all studies

Direction of change and effect sizes for serum P, Ca, PTH, and urinary P is shown for all studies. The same values were reported for Aparicio et al. (1992)[329] and Combe et al. (1993)[328] for CKD-MBD outcomes. Thus, to prevent reporting bias, only results from Combe et al. (1993)[328] are presented. Horizontal arrows indicate no change, up arrows indicate an increase, and down arrows indicate a decrease in the outcome reported. Effect size is shown by arrow number with one arrow representing a small magnitude of effect, two arrows representing a

medium magnitude of effect, and three arrows representing a large magnitude of effect. White arrows were used when effect size was unable to be determined. *NR* not reported, *prt* protein, *E* endpoint, *B* baseline, *LPD* low protein diet, *veg* vegetarian *MD* Mediterranean diet, *HBFR* high bone formation rate, *NBFR* normal bone formation rate, *LBFR* low bone formation rate, *FD* free diet, *VLPD-C* very low protein diet compliant, *VLPD-NC* very low protein diet non-compliant. [a]No statistical tests were performed. Thus, statistical differences in outcomes were not reported for this study and true direction of changes unable to be determined. [b]Decreased in meat group compared with vegetarian group. [c]Decreased in LPD to a greater extent than VLPD, but also decreased from baseline to 12 months on the VLPD compared with baseline.[d]Decreased in those with LBFR on VLPD compared to baseline. [e]Decreased in those with NBFR and LBFR on VLPD compared to baseline. All reported values for outcomes can be found in **Supplementary File 4** (https://www.doi.org/10.17605/OSF.IO/YFR57).

Appendix E: National and International Abstracts and Posters

Appendix E.1: Burstad, KM; Fons, A; Kisch, AR; Cladis, DP; Hill Gallant, KM; Phosphorus Bioaccessibility of Milk and Beef Plant-Based Protein Alternative Products using In Vitro Simulation of Human Digestion. *National Kidney Foundation Spring Clinical Meeting 2023*, Austin, TX, April 11-15

Plant-based protein (PBP) is of growing interest for patients with CKD because of several proposed benefits, including the potentially lower phosphorus (P) bioaccessibility from PBP compared with animal proteins. However, P bioaccessibility data are sparse and are absent for emerging PBP products. Here we determined P bioaccessibility of PBP alternatives to conventional milk and beef products using in vitro simulation of human digestion. Animal and PBP milk (n=7) and beef (n=11) products were evaluated. Products were prepared according to package directions and freeze dried prior to in vitro experiments, which consisted of oral, gastric, and intestinal phases to simulate human digestion. The final digesta were dialyzed against pure water across a 500-1000 MWCO membrane for 30h to allow for equilibration of bioaccessible forms of P. P content was analyzed in the pre-digestion, post-digestion, and dialysate samples by MP-AES to calculate total and bioaccessible P. For PBP milk alternatives, soy protein products had the lowest bioaccessible P, which averaged 46mg P/100g and was comparable to cow's milk (55mg P/100g), but pulse protein products had >3x the bioaccessible P (183mg P/100g). % bioaccessibility was 47, 73, and 94%, respectively. Bioaccessible P to protein ratio was lowest in soy (9.7mg/g), followed by animal (16.1mg/g), and highest in pulse protein products (78.4mg/g). For PBP beef alternatives, soy products averaged 89mg P/100g and were similar

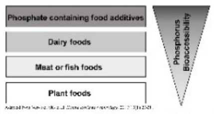
to pulse products, which averaged 76 mg P/100g, both of which were lower than bioaccessible P in beef averaging 140mg P/100g. % bioaccessibility was 51, 29, & 84%, respectively. Bioaccessible P to protein ratio was 3.9mg/g in pulse and 4.6mg/g in soy products, about half that of animal products (8.4 mg/g). Overall, the soy and pulse PBP alternatives offered lower bioaccessible P per 100g serving and per g of protein, except for the pulse protein milk alternatives which contained high bioaccessible P. These findings indicate that some processed PBP products may be suitable for inclusion in dietary recommendations for P management in patients with kidney disease.

Phosphorus Bioaccessibility of Milk and Beef Plant-Based Alternative Products Using *In Vitro* Simulation of Human Digestion

Kendal M. Burstad, RDN[1], Alexandria Fons, MS, RDN[1], Abigail R. Kisch[1], Dennis P. Cladis, PhD[1], Kathleen M. Hill Gallant, PhD, RD[1]
[1]Department of Food Science and Nutrition, University of Minnesota

Introduction

- Current clinical guidelines encourage taking phosphate source into consideration when making dietary recommendations.
  - (1) **KDOQI 2020 (6.3.2)**: It is reasonable when making decisions about phosphorus restriction treatment to consider the bioavailability of **phosphorus sources** (**e.g. animal, vegetable, additives**). *(Opinion)*
  - *(2)* **KDIGO 2017 (4.1.8):** In patients with CKD G3a-G5D, we suggest limiting dietary phosphate intake in the treatment of hyper-phosphatemia alone or in combination with other treatments (*2D*). It is reasonable to consider **phosphate source** (**e.g., animal, vegetable, additives**) in making dietary recommendations (*Not Graded*).



Phosphorus bioaccessibility is thought to be lowest from plant-foods followed by animal foods and is highest in inorganic phosphate containing food additives.

- The KDOQI 2020 report provides additional commentary to choose natural foods that are lower in bioaccessible phosphorus. **However, little data is available on phosphorus bioaccessibility of food products.**
- In addition, there has been an increase in the development and availability of processed plant-based protein alternatives to animal-based protein products. **But virtually no data is available on phosphorus bioaccessibility of these new emerging plant-based products.**

Aim: To determine phosphorus bioaccessibility of emerging plant-based protein products in comparison to their animal protein counterparts using *in vitro* simulation of human digestion.

Materials and Methods

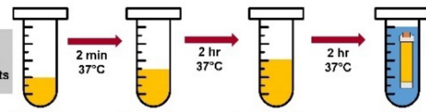
Product Selection → **Product Preparation** → **Product Freeze Drying**

n=7 soy-based; n=8 pulse-based
n=3 animal-based; 2 food categories

Prepared according to package directions

Freeze dried for 4-7 days

***In Vitro* Digestion Experiments**



→ 2 min 37°C → 2 hr 37°C → 2 hr 37°C → 30 hr ~20-22°C Room Temp. → **Mineral Analysis via MP-AES**

Oral Phase: Simulated Salivary Fluid; Salivary Amylase

Gastric Phase: Simulated Gastric Fluid; Pepsin; pH 3

Intestinal Phase: Simulated Intestinal Fluid; Pancreatin; Bile; pH 7

Dialysis: 5 mL diluted digesta; 40 mL ultrapure water; 500-1000 MWCO membrane

Phosphorus analyzed in pre-digestion, post-digestion and dialysate

Analysis: Data were analyzed using Microsoft® Excel® (Version 2302). Descriptive statistics were used to characterize and compare data among protein sources (soy, pulse, and animal) and within each food category (beef and milk). Regression analysis was performed to determine the relationship between percent and absolute bioaccessible phosphorus for each food category. Mean values for each product are shown.

Results

Figure 1: Phosphorus bioaccessibility of beef and beef alternative products

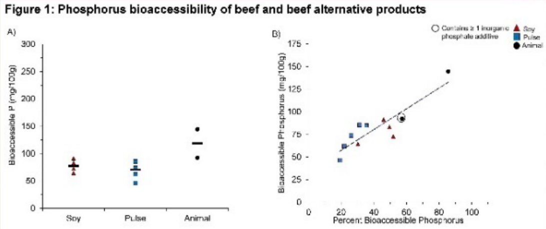


Figure 1: A) Average absolute bioaccessible phosphorus was similar between pulse- and soy-based beef (71 mg/100g and 78 mg/100g). Both pulse- and soy-based beef had lower absolute bioaccessible phosphorus than animal-based beef (119 mg/100g). **B)** Average percent bioaccessible and absolute bioaccessible phosphorus was lower for pulse- and soy-based beef compared to animal-based beef. There was a strong correlation between the percent bioaccessible and absolute bioaccessible phosphorus for the beef and beef alternative products evaluated (R^2=0.79, p=0.0002).

Figure 2: Phosphorus bioaccessibility of milk and milk alternative products

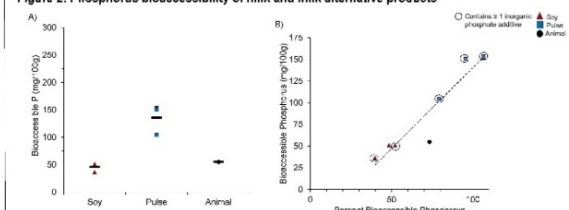


Figure 2: A) Average absolute bioaccessible phosphorus was lowest in soy-based milk (46 mg/100g) but was comparable to cow's milk (55 mg/100g). Average absolute bioaccessible phosphorus for pulse-based milk was ~3x that of soy-based and cow's milk. **B)** Average percent bioaccessible phosphorus was highest in pulse-based milk and lowest in soy-based milk. There was a strong correlation between the percent bioaccessible and absolute bioaccessible phosphorus for milk and milk alternative products (R^2=0.88, p=0.002). Additionally, in reviewing the Nutrition Facts Labels, it was observed that soy-based milk had a lower bioaccessible phosphorus-to-protein ratio (9.7 mg/g) compared with cow's milk (16.1 mg/g).

- Soy- and pulse-based beef and soy-based milk protein products offered lower bioaccessible phosphorus per 100g serving than their animal counterparts.
- Soy-based milk may be a better option for patients with CKD on dialysis due a more optimal bioaccessible phosphorus-to-protein ratio than cow's milk.

Certain plant-based products may be suitable for inclusion into the diets of patients with CKD but more data on phosphorus bioaccessibility is needed to better inform individualized guidance

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Contact information:
Kendal Burstad, RDN | PhD Candidate – Nutrition, University of Minnesota | schm3950@umn.edu

University of Minnesota
Driven to Discover

Appendix E.2: Burstad, KM; Fons, A; Hill Gallant K., Phosphorus Content and Phosphorus-to-Protein Ratio Among Plant-Based Protein Products, *American Society of Nephrology Kidney Week 2022*, Orlando, FL, November 3-6.

Plant-based eating is of growing interest in management of CKD due to several reasons, including the proposed lower P bioavailability from plant sources. However, few data are available on the P content of emerging plant-based products. In this study, we aimed to quantify P in several popular food categories of plant-based foods (soy or other pulse-based) and compared to their animal protein counterparts. Our results for plant-based dairy and ground beef alternatives were presented at the National Kidney Foundation Spring Clinical Meeting 2022 and overall showed that P content and P-to-protein ratio were lower in soy- compared to pulse-based products, and soy products were comparable to their animal protein counterparts.

Here, we present results for plant-based chicken/turkey, sausage/bacon, yogurt/cheese, and other popular soy/pulse products. Products were prepared according to package directions, freeze-dried, ashed and analyzed for P content using MP-AES.

Analyzed P content ranged from 116-196 mg P/100g, 80-293 mg P/100g, and 5-346 mg P/100g for plant-based chicken/turkey, bacon/sausage, and yogurt/cheese products, respectively. For comparison, P content from animal sources in the categories of chicken/turkey, bacon/sausage, and yogurt/cheese ranged from 125-273 mg P/100g, 122-237 mg P/100g, and 105-1223 mg P/100g.

Analyzed P content of other soy products (i.e., tofu, tempeh, etc.) ranged from 145-571 mg P/100g and of other pulse products (i.e., chickpea, green lentils, etc.) ranged from 52-166 mg P/100g. Nine of the 40 products analyzed had least one inorganic phosphate additive listed on the label. Total P content of plant-based chicken/turkey alternatives was lowest in pulse-based products, but the P-to-protein ratio was lowest in a soy-based chicken product (3.8 mg/g). Total P content of soy-based cheese products was lower than pulse-or animal-based. However, these soy-based cheeses contained no protein, and animal- and pulse-based cheese products had the highest P-to-protein ratios of all food studied, 85.6 mg/g and 103.6 mg/g.

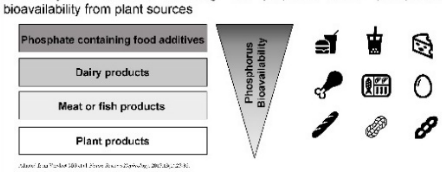
These data show the wide variation in P content and P-to-protein ratio of both plant-based products and their animal-based counterparts. Further quantification and reporting of P content in emerging plant-based products is needed for appropriate recommendations for patients with CKD.

Phosphorus Content and Phosphorus-to-Protein Ratio Among Plant-Based Protein Products

Kendal M. Burstad,[1] Alexandria Fons[1], Kathleen M. Hill Gallant[1]
[1]Department of Food Science and Nutrition, University of Minnesota

Introduction

Plant based eating is a growing interest for dietary management of CKD for a variety of reasons, including the proposed lower phosphorus bioavailability from plant sources



- **KDOQI 2020 (6.3.2):** It is reasonable when making decisions about phosphorus restriction treatment to consider the bioavailability of **phosphorus sources (e.g. animal, vegetable, additives)**. *(Opinion)*
- **KDIGO 2017 (4.1.8):** In patients with CKD G3a-G5D, we suggest limiting dietary phosphate intake in the treatment of hyper-phosphatemia alone or in combination with other treatments (2D). It is reasonable to consider phosphate source (e.g., animal, vegetable, additives) in making dietary recommendations *(Not Graded)*.
- Few data are available on the phosphorus content of emerging plant-based products and phosphorus content is not required on the nutrition facts label.
- In addition, databases are incomplete and often underestimate phosphorus. Phosphorus has been found to be underestimated by **~40%** in a mixed meal[1] and by **15-20%** in various food products.[2]

What is the phosphorus content of plant-based foods (soy-or pulse-based) from popular food categories?

Materials and Methods

Protein Products: Three categories of proteins were compared (1) pea-based (2) soy-based, and (3) animal based. Plant-based chicken/turkey, sausage/bacon, yogurt/cheese, and other popular soy and pulse products were selected based on popular demand sorted by "best seller" filters and retail sales data from the Good Foods Institute and SPINS to ensure that products were representative of what is being consumed in American diets. Products were purchased from Target, Walmart, and Whole Foods located in Minnesota. All products were prepared according to package directions.

Product Selection → **Product Preparation** → **Product Freeze Drying** → **Product Ashing** → **Mineral Analysis via MP-AES**

Freeze Drying: Products were cooled to -20°C then placed in the freeze dryer for 5-7 days.

Muffle Furnace: Freeze dried products were ashed at 300°C for 16 hours followed by 600°C for 72 hours. Ash was dissolved and diluted in 70% nitric acid and analyzed for mineral content via microwave plasma – atomic emission spectrometry (MP-AES).

Analysis: Qualitative comparisons were made between soy and pulse-based products and their animal protein counterparts for phosphorus content and phosphorus-to-protein ratio. Mean values for each product are shown.

Results

Figure 1. Phosphorus mg/100g of plant-based chicken/turkey, sausage/bacon, yogurt/cheese, and other popular soy and pulse products

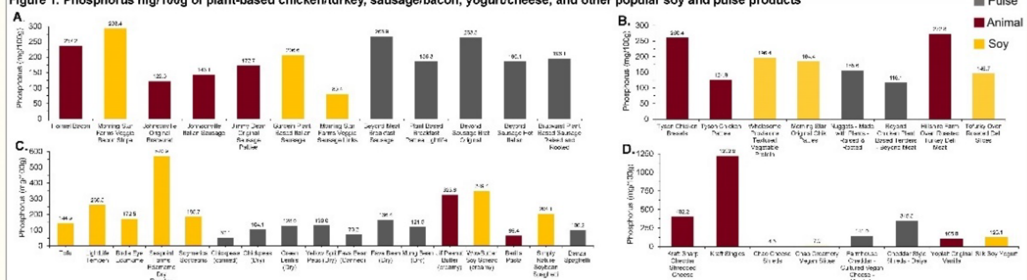


Figure 1. Phosphorus mg/100g of plant-based chicken/turkey, sausage/bacon, yogurt/cheese, and other popular soy and pulse products. Chemically analyzed P ranged from A) 80-293 mg P/100g for plant-based bacon/sausage and 122-237 mg P/100g for bacon/sausage animal products. B) 116-196 mg P/100g for plant-based chicken/turkey and 125-273mg P/100g chicken/turkey animal products C) 145-571 mg P/100g for soy products including tofu, tempeh, etc. and 52-166 mg P/100g for pulse products including chickpeas, lentils, etc. D) and 5-348 mg P/100g plant-based yogurt/cheese products and 105-1223 mg P/100g for yogurt/cheese animal products. The total P content of chicken/turkey was lowest in a pulse-based product while the total P content of bacon/sausage and yogurt/cheese was lowest in soy-based products.

Figure 2. Phosphorus-to-protein ratio mg P/g protein of plant-based sausage/bacon, chicken/turkey, other popular soy and pulse products, and yogurt/cheese

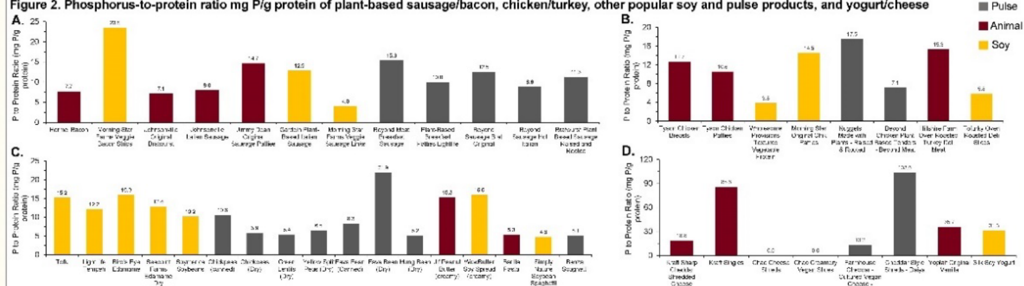


Figure 2. Phosphorus-to-protein ratio mg P/g protein of plant-based chicken/turkey, sausage/bacon, yogurt/cheese, and other popular soy and pulse products. P-to-protein ratio ranged from A) 4-23.5 mg P/g for plant-based bacon/sausage and 7.1-14.7 mg P/g for bacon/sausage animal products. B) 3.8-17.5 mg P/g for plant-based chicken/turkey and 10.6-15.3 mg P/g chicken/turkey animal products C) 4.8-16 mg P/g for soy products including tofu, tempeh, etc. and 5.1-21.9 mg P/g for pulse products including chickpeas, lentils, etc. D) and 0-103.6 mg P/g plant-based yogurt/cheese products and 18.8-85.6 mg P/100g for yogurt/cheese animal products. Animal- and pulse-based cheese products had the highest P-to-protein ratios of all the foods studied whereas the soy-based cheeses contained no protein. The lowest P-to-protein ratio was in a soy-based chicken product.

These data show the wide variation in P content and P-to-protein ratio of both plant-based products and their animal-based counterparts.

Further quantification and reporting of P content in emerging plant-based products is needed to make appropriate recommendations for patients with CKD.

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Contact information:
Kendal Burstad, RDN | PhD Candidate – Department of Food Science and Nutrition, University of Minnesota | schm3950@umn.edu

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Appendix E.3: Burstad, KM; Cladis, DP; Wiese, GW; Butler, M; Hill Gallant K., Effects of Plant-Based Protein Consumption on Kidney Function and Mineral Bone Disorder Outcomes in Stage 3-5 CKD Patients: A Systematic Review, 20th Congress of the International Society of Renal Nutrition and Metabolism, Guangzhou, China, June 16-18 2022.

Plant-based protein consumption is of growing interest for dietary management of chronic kidney disease and may lead to preserving kidney function and reducing CKD-mineral bone disorder (CKD-MBD) complications. This systematic review aimed to summarize the available evidence for the effect of plant-based protein on kidney function and CKD-MBD outcomes in stage 3-5 CKD patients not on dialysis.

We searched Medline, Embase, Agricola, and CAB Abstracts via Ovid, Web of Science, and Scopus, along with handsearching, through July 2021. We included clinical trials with ≥8 participants ≥18 years of age with an eGFR <60 mL/min/1.73m^2 but not on dialysis. Additionally, we required an intervention period of ≥1 week with ≥50% dietary protein from plant-based sources and at least one outcome for both kidney function and CKD-MBD. Only English-language studies published in peer-reviewed journals were included, though grey literature was included if it had sufficient information to assess eligibility and risk of bias. We assessed risk of bias using the ROB-2 tool for randomized and crossover trials. Analysis was limited to qualitative synthesis as the heterogeneity of included studies prevented quantitative meta-analysis.

Thirty-two studies with a cumulative 1,182 participants were included of which, 1 was a case control trial, 4 were crossover trials, 16 were single arm, and

11 were parallel arm. Study durations ranged from 1 week to 65 months. Most studies (27/32) investigated the effect of a change in protein amount (typically, ~0.3-0.4 g/kg/d with ketoanalogue supplementation) in tandem with a change in protein source from animal to plant-based. This limits our ability to distinguish effects of protein source from effects of protein amount in these studies. However, a subset of 5 studies investigated a change in protein source from animal to plant-based as the main intervention while keeping total protein amount constant. Of this subset, 4 studies reported no change in CrCl or eGFR while 1 study reported a significant decrease in CrCl. Three studies reported no change in serum phosphorus and 1 study reported significantly lower serum phosphorus levels. Further, inconclusive results were observed for the phosphaturic hormones, PTH and FGF-23. Overall, evidence from the included studies was of suboptimal methodological quality and yielded inconclusive results, thus concrete conclusions of the effects of plant-based protein consumption on kidney function and CKD-MBD outcomes cannot be made. Further research on the effects of protein source on these outcomes in CKD is required.

ISRNM2022 20TH CONGRESS OF THE INTERNATIONAL SOCIETY OF RENAL NUTRITION AND METABOLISM

Effects of Plant-Based Protein Consumption on Kidney Function and Mineral Bone Disorder Outcomes in Stage 3-5 CKD Patients: A Systematic Review

Kendal M. Burstad,[1] Dennis P. Cladis,[1] Gretchen N. Wiese,[2] Mary Butler[3], Kathleen M. Hill Gallant[1,4]

[1]Department of Food Science and Nutrition, University of Minnesota; [2]U.S Renal Care, Altitude Dialysis, Aurora, Colorado; [3]Division of Health Policy and Management, University of Minnesota School of Public Health; [4]Department of Medicine-Division of Nephrology, Indiana University School of Medicine

Background:

Plant-based protein consumption is of growing interest for dietary management of chronic kidney disease (CKD) and may lead to preserving kidney function and reducing CKD-mineral bone disorder (CKD-MBD) complications.

What is the effect of incorporation of plant-based proteins on kidney function and CKD-MBD outcomes in stage 3-5 CKD patients?

Methods:

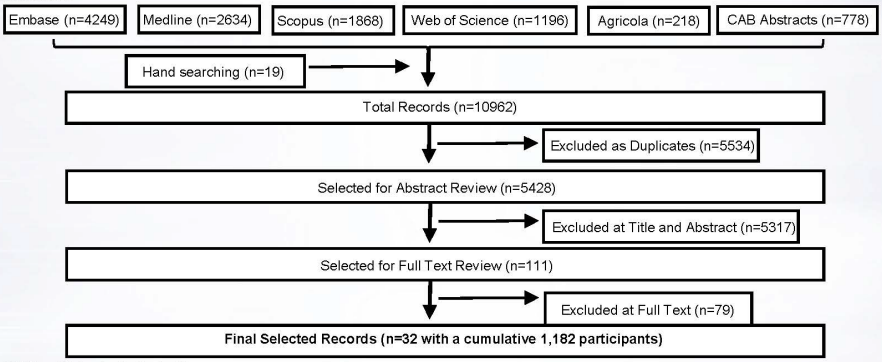
We searched Medline, Embase, Agricola, and CAB abstracts via Ovid, Web of Science and Scopus along with handsearching through July 2021.

Inclusion Criteria:

- Clinical trials with ≥8 participants ≥18 years of age with an eGFR <60 mL/min/1.73m^2 and not on dialysis.
- An intervention period of ≥1 week with ≥50% dietary protein from plant-based sources and at least one outcome for both kidney function and CKD-MBD.
- Only English-language studies published in peer-reviewed journals were included, though grey literature was included if it had sufficient information to assess eligibility and risk of bias.

We assessed risk of bias using the ROB-2 tool for randomized and crossover trials. Analysis was limited to qualitative synthesis as the heterogeneity of included studies prevented quantitative meta-analysis.

Results:



Of the 32 included studies:

- **Study Type:** Case control trial (n=1), crossover trials (n=4), single arm (n=16), parallel arm (n=11)
- **Study Duration:** ranged from 1 wk to 65 mos
- **Study Intervention:** n=27 investigated the effect of a change in protein amount (typically, ~0.3-0.4 g/kg/d with ketoanalogue supplementation) in tandem with a change in protein source from animal to plant-based. This limits our ability to distinguish effects of protein source from effects of protein amount in these studies.

n=5 investigated a change in protein source from animal to plant-based as the main intervention while keeping total protein amount constant. Thus, effects of protein source are able to be distinguished. These data are shown below.

Study	Duration	N	Treatment	CrCl or eGFR (mL/min/1.73m^2)	Proteinuria (g/24 hr)	Serum P (mg/dL)	Serum PTH (pg/mL)
Moe 2011	1 wk/diet	8	Veg. vs meat	→	—	↓↓↓	★↓
Moorthi 2014	4 wks	13	70% plant prt	→	—	→	→
Fois 2019	≥ 3 mo	65	LPD veg. vs mix	⇢	⇢	—	⇢
Soroka 1998	6 mo	9	Veg. vs mix	→ → →	→	→ →	—
Barsotti 1996	12.8 ± 5.7 mo	11	LPD mixed to veg	⋮↓	—	⇢	—

Direction of change for kidney function and CKD-MBD outcomes are shown. Four studies reported no change in CrCl or eGFR while 1 study reported a significant decrease in CrCl. Three studies reported no change in serum phosphorus and 1 study reported significantly lower serum phosphorus levels. Horizontal arrows indicate no change, up arrows indicate an increase, and down arrows indicate a decrease in the outcome reported. Effect size is shown by arrow number 1 arrow (small), 2 arrows (medium), 3 arrows (large). Arrows with dotted lines were used when effect size was unable to be determined. – Outcome not reported
★ PTH was significantly lower in those in the meat group compared to the veg. veg = vegetarian diet, meat = animal-based protein diet, mix = mixed protein diet, LPD = low protein diet, prt = protein

Overall, evidence from the included studies was of suboptimal methodological quality and yielded inconclusive results, thus concrete conclusions of the effects of plant-based protein consumption on kidney function and CKD-MBD outcomes cannot be made.

Further research on the effects of protein source on these outcomes in CKD is required.

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JUNE 16-18 (THU-SAT), 2022 | GUANGZHOU, CHINA

Appendix E.4: Burstad, KM; Fons, A; Hill Gallant K., Phosphorus Content and Variation among Plant-Based Beef Alternative Products, National Kidney Foundation Spring Clinical Meeting 2022, Boston, MA, April 6-10. Poster #378

Plant-based eating is of growing interest for dietary management of CKD. A proposed benefit of plant-based eating is lower bioavailability of phosphorus (P) from plant sources. However, P content is not required on the Nutrition Facts label, and nutrient databases are incomplete and often underestimate P. Consumer demand for plant-based meat alternatives is rising, but few data are available on the P content of these emerging plant-based products.

Here, we quantified P in plant-based ground beef alternatives, and compared to food label and nutrient database values, when available. 8 plant-based ground beef alternative (soy or pea protein) products were evaluated, along with ground beef for comparison. Products were prepared according to package directions, freeze dried, ashed, and analyzed for P content using ICP-OES. Nutrient database P values were obtained from USDA FoodData Central, and food labels evaluated for inclusion of P content and additive information. All products were first searched for a brand match, and if unavailable, were searched for a best generic match.

Analyzed P content ranged from 197-388 mg P/100g among the plant-based products evaluated. For comparison, ground beef had 210-221 mg P/100g. Nutrient database content was available for only 1 product (ImpossibleTM soy burger patties), which underestimated the analyzed P content

by ~33%. The remaining plant-protein products were compared with the best generic match: P content of 6 products was underestimated in the database by ~16-47%, and 1 product was slightly overestimated by ~4%, compared with analyzed values. None of the plant-based products in this study listed phosphate additives on the label. P-to-protein ratio of the plant-based ground beef alternatives ranged from 8-21 mg P/g of protein, compared to 13 mg/g for ground beef (animal). P-to-protein was lowest in a soy burger (MorningStar Farms® Vegan Meat Lovers, 8 mg/g). P-to-protein was highest in pea-protein ground beef patty alternatives, ranging from 16-21 mg/g. These results show variation in P content and P-to-protein among plant-based beef alternatives made from soy and pea protein and underscores the need for analysis and reporting of P content in emerging plant-based products.

Phosphorus Content and Variation among Plant-Based Beef Alternative Products

Kendal M. Burstad,[1] Alexandria Fons[1], Kathleen M. Hill Gallant[1]
[1]Department of Food Science and Nutrition, University of Minnesota

Introduction

Figure 1. Dietary phosphorus can have negative health consequences for chronic kidney disease patients.
As kidney function declines, so does the kidney's ability to excrete phosphorus (P). This leads to increased serum P, PTH, and FGF23, ultimately leading to increased CVD and fracture risk. Thus, dietary management is aimed at restricting phosphorus consumption.

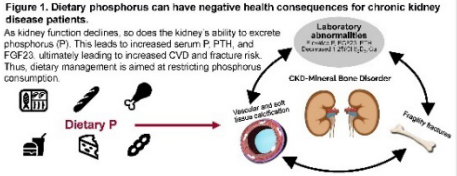
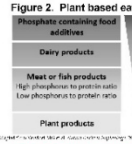


Figure 2. Plant based eating is a growing interest for dietary management of CKD



KDOQI 2020 (6.3.2): It is reasonable when making decisions about phosphorus restriction treatment to consider the bioavailability of **phosphorus sources (e.g. animal, vegetable, additives)**. *(Opinion)*

A proposed benefit of plant-based eating is the lower bioavailability of P from plant sources. However, P content is not required on the Nutrition Facts label and nutrient databases are incomplete and often underestimate P.

- P underestimated by **~40%** in a mixed meal[1] and by **15-20%** in various food products.[2]

What is the P content of various emerging plant-based beef alternatives and how does this compare to nutrient database values?

Materials and Methods

Product Procurement and Processing Steps

Product Selection → Product Preparation → Product Freeze Drying → Product Ashing → Mineral Analysis via ICP-OES

Protein Products:
Three categories of proteins were compared (1) pea-based (2) soy-based, and (3) animal based. Beef and beef alternative products selected were based on popular demand sorted by "best seller" filters and retail sales data from the Good Foods Institute and SPINS to ensure that products were representative of what is being consumed in American diets. Products were purchased from Target, Walmart, and Whole Foods located in Minnesota. All products were prepared according to package directions.

Figure 3. Soy- and pea-based beef and beef alternative products

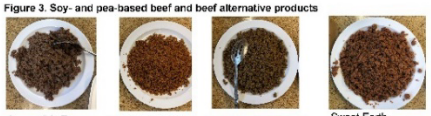


Figure 3. Soy- and pea-based beef and beef alternative products. Two soy-based (Impossible burger and Gardein Ground Be'f) and two pea-based (Beyond Meat Beyond Beef Crumbles and Sweet Earth Awesome Burger) are shown.

Materials and Methods

Product Processing:

Freeze Drying: All products were weighed prior to and following completion of freeze drying. Products were first place in the -20°C and once they were cooled were placed in the freeze dryer for 5-7 days.

Muffle Furnace: All products were weighed prior to the ashing procedure. Products were ashed at 300°C for 16 hours followed by 600°C for 72 hours. Products were then prepared for mineral analysis via inductively coupled plasma – optical emission spectrometry (ICP-OES) by dissolving and diluting product ash in 2% nitric acid.

Nutrient Database:
Nutrient database phosphorus content was obtained from USDA FoodData Central, and food labels were evaluated for inclusion of P content and additive information. All food products were first searched for a brand match in FoodData Central and if unavailable, were searched for a best generic match.

Analysis:
Qualitative comparisons were made between nutrient database and chemically analyzed phosphorus content of beef alternative products and between soy and pea-based beef alternatives and ground beef (animal) products for phosphorus content and phosphorus-to-protein ratio. Mean values for each product are shown.

Results

Figure 4: Phosphorus mg/100 g from soy, pea, and animal-based beef and beef alternative products

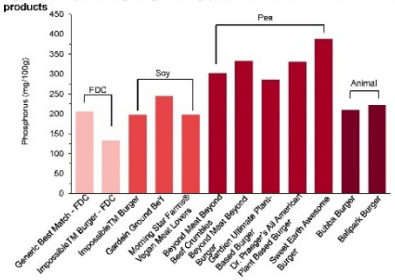


Figure 4: Phosphorus mg/100 g from soy, pea, and animal-based beef and beef alternative products. Chemically analyzed P content ranged from 197-388 mg P/100g among soy and pea plant-based beef products. P values were lower in soy-based products compared to pea-based products. Chemically analyzed P content ranged from 210-221 mg P/100g beef (animal) products. Nutrient database P content was available for only 1 product (Impossible Burger soy-based patties) and was underestimated by ~33%. All other products were compared to a best generic match in which 6 products were underestimated by ~16-47% and 1 product was overestimated by ~4%.

Results

Figure 5: Phosphorus-to-protein ratio of soy, pea, and animal-based beef and beef alternative products

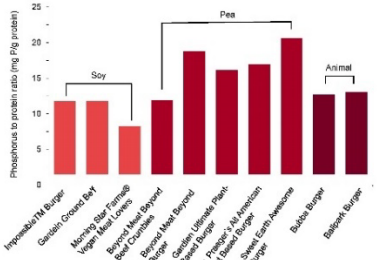


Figure 5: Phosphorus-to-protein ratio of soy, pea, and animal-based protein products
Phosphorus-to-protein ratio ranged from 8-12 mg/g in soy-based protein products and 12-21 mg/g in pea-based protein products compared to 13 mg/g for beef (animal) products.

Taken together, these results show variation in total P and P-to-protein ratio among beef alternative products. Further, better total P and P-to-protein profiles were observed for soy compared to pea protein beef alternatives.

This underscores the need for analysis and reporting of P content in emerging plant-based products to aid in optimal recommendations for patients with CKD.

References

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Contact information:
Kendal Burstad, RDN | PhD Candidate – Department of Food Science and Nutrition, University of Minnesota | schm3850@umn.edu

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Appendix E.5: Schmitz, KM; Cladis, DP; Vorland, CJ; Biruete, A; O'Neill, K; Chen, NX; Moe, SM; Hill Gallant, KM. 2021. Effects of Low and High Dietary Phosphorus and Acute High Dietary Phosphorus on Intestinal Phosphate Transporters and Biochemistries in Nephrectomized Male Rats. American Society for Bone and Mineral Research Nat'l Mtg, San Diego, CA, Oct 1-4. ePoster #VPL-399

Dietary phosphorus(P) restriction and P binders are commonly used in patients with chronic kidney disease(CKD) to reduce intestinal P absorption to slow development of CKD-mineral and bone disorder. We previously reported that *in vivo* P absorption efficiency is lower when an acute high P load is given following a week of low P diet in male rats with and without CKD(ASBMR 2020 #742). Here, we report further data from this study on the effects of dietary P level and acute high P on plasma P, phosphaturic hormones, and intestinal P transporter expression.

Male CD(SD) rats with 5/6 nephrectomy (n=36) or sham (n=36) were block randomized to 1 of 3 diets: low P(LP, 0.1%), high P(HP, 1.2%), or low P followed by acute high P(LPHP, 0.1% then 1.2%)(n=12/group) and were fed for 4h/d for 7d. LPHP rats were fed the LP diet on days 1-6 and the HP diet on day 7. *In vivo* intestinal P absorption was measured by oral gavage. After sacrifice, intestinal brush border membrane was collected and analyzed for gene expression of duodenal and jejunal Napi2b, PiT1, and PiT2. Plasma was analyzed for P, Ca, iPTH, and iFGF23. A linear mixed model was used to determine main effects of diet, disease, and their interaction.

Rats on either HP or LPHP diets had higher plasma P compared with rats on LP diet, and this was seen in both CKD and sham rats, but to a greater magnitude in CKD rats. Further, CKD rats on the LPHP diet had higher plasma P compared to sham rats on the LPHP diet(disease*diet $p<0.0001$). Plasma Ca was lower in rats on the LPHP diet compared with rats on either LP and HP diets(diet main effect $p<0.0001$). iFGF23 was lowest in both CKD and sham rats on the LP diet compared with the HP and LPHP diets, but CKD rats on the HP diet had ~2x higher levels than any other group(disease*diet $p=0.006$). iPTH was lowest in rats on the LP diet, followed by the LPHP diet, and highest in rats on the HP diet(diet main effect $p<0.0001$). Duodenal PiT2 mRNA was significantly higher in LPHP rats compared with either LP or HP rats(diet main effect $p=0.0002$). Jejunal Napi2b mRNA was significantly lower in LPHP rats compared with rats on the HP diet(diet main effect $p=0.01$).

These results show that the lower intestinal P absorption we previously observed in response to acute high P following a week of low P diet is accompanied by higher plasma iFGF23 and iPTH and lower expression of jejunal Napi2b mRNA, yet this was not sufficient to prevent elevated plasma P in response to the acute P load.

ASBMR 2021 ANNUAL MEETING

ASBMR The American Society for Bone and Mineral Research

October 1 – 4, 2021

Effects of Low and High Dietary Phosphorus and Acute High Dietary Phosphorus on Intestinal Phosphate Transporters and Biochemistries in Nephrectomized Male Rats

K.M. Schmitz,[1] D.P. Cladis,[1] C.J. Vorland,[2] A. Biruete,[3] J. Dominguez,[3] K. O'Neill,[3] N.X Chen,[3] S.M. Moe,[3] K.M. Hill Gallant[1]

[1]Department of Food Science and Nutrition, University of Minnesota [2]Department of Applied Health Science, Indiana University School of Public Health-Bloomington, [3]Department of Medicine-Division of Nephrology, Indiana University School of Medicine

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INTRODUCTION

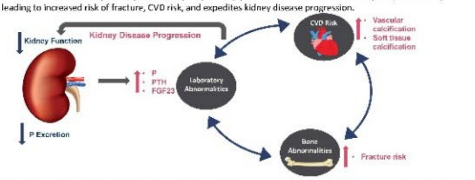


Figure 1. Dietary phosphorus can have negative health consequences for chronic kidney disease (CKD) patients. As kidney function declines so does the kidney's ability to excrete phosphorus (P). This leads to increased serum P, PTH, and FGF23, ultimately leading to increased risk of fracture, CVD risk, and expedites kidney disease progression.

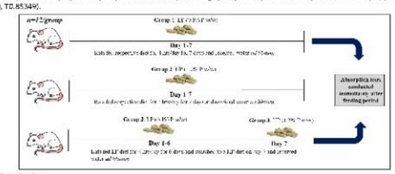
Depending on stage of CKD, recommendations for CKD patients are to follow a low phosphorus (LP) diet or take P binders because of the implications a high phosphorus (HP) diet can have on serum P, kidney function, bone, and CVD risk. However, bouts of non-adherence to the diet are common. Giral et al. have reported that acute P loading following adaptation to a low P diet in healthy rats produced a maladaptive effect where intestinal P absorption efficiency was enhanced and serum P elevated.

Conversely, we previously reported that *in vivo* P absorption efficiency is lower when an acute high P load is given following a week of low P diet in male rats with and without CKD. Here, we report further data from this study on the effects of dietary P load and acute high P on plasma P, phosphaturic hormones and intestinal P transporter expression. ASBMR 2020 #742

MATERIALS & METHODS

Design:

- This study was a 2x3 factorial design in which 7-8 week old male Sprague-Dawley (CD) rats underwent a 2-step 5/6th nephrectomy (n=36) or sham operation (n=36) at Charles River Laboratories.
- After a 1-week recovery period, rats were shipped to Purdue University and fed a standard rodent chow (Harlan Teklad 2018, Indianapolis, IN) for 3 weeks. At 4 weeks post-surgery, rats were switched to a randomly assigned diet (block randomized by time) treatment of either low phosphorus (LP 0.1% P w/w), high phosphorus (HP 1.2% P w/w), or Low phosphorus followed by acute high phosphorus (LPHP 0.1% P w/w then 1.2% P w/w) (Envigo Teklad, Indianapolis, IN: TD.85010, TD.85349).



Plasma Biochemistries:

- Sacrifice blood draws were taken, centrifuged for plasma, and stored at -80°C until analysis.
- Plasma was analyzed for phosphorus and calcium using standard clinical chemistries and intact parathyroid hormone (iPTH),and intact fibroblast growth factor 23 (iFGF23) using enzyme-linked immunosorbent assay (ELISA) (BioAssay Systems, Hayword, CA, USA, Point Scientific, Canton, MI, USA, and Quidel Corporation, San Diego, CA, USA).

Intestinal Phosphate Gene Expression:

- Total RNA from duodenum and jejunum was isolated using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Target-specific PCR primers were obtained from Applied Biosystems (Foster City, CA, USA): NaPi2b (Slc34a2; Rn00584515_m1); PiT1 (Slc20a1; Rn00579811_m1); ribosomal protein, large, P0 (RPLP0, Rn03302271_gH). The gene expression was analyzed by real-time PCR using TaqMan gene expression assay system (TaqMan MGP probes, FAM dye-labeled; Applied Biosystems, Foster City, CA, USA) using ViiA 7 systems. Using the ΔΔCT method to assess the relative changes in mRNA expression and normalized by RPLP0. (Chen, et al, J Bone Miner Res. 2020 Mar;35(3):608-617)

Statistics:

- A linear mixed model was used to determine main effects of diet and disease and the interaction.
- Rats with outliers in plasma BUN were thoroughly investigated and removed from all analysis if they had a student deleted residual (SDR) ≥2.7. Removed n=5 rats (3 sham HP, 1 sham LP, and 1 CKD HP).

RESULTS

Figure 2: Plasma Biochemistries

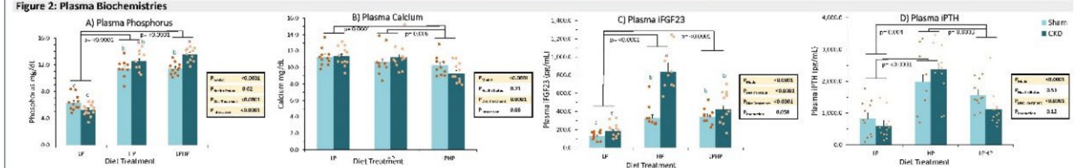


Figure 2. Plasma Biochemistries: A) For both CKD and sham rats, plasma P was higher with LPHP and HP diets compared to the LP diet. Within diets, CKD rats had higher plasma P compared to sham only on the LPHP diet ($P_{Interaction}$ = <0.0001). **B)** Rats on the LPHP diet had lower plasma calcium ($P_{Diet\ Treatment}$ = 0.0001). **C)** iFGF23 was lowest in both CKD and sham rats on the LP diet compared with the HP and LPHP diets. Additionally, CKD rats on the HP diet had ~2x higher levels (837.15 ± 100.7 pg/mL) compared to any other group (CKD LPHP 419.89 ± 42.06 pg/mL, sham HP 327.01 ± 39.34 pg/mL, sham LPHP 340.36 ± 37.52 pg/mL). **D)** Plasma iPTH was lowest in rats on the LP diet, followed by the LPHP diet, and highest in rats on the HP diet ($P_{Diet\ Treatment}$ =<0.0001). Different letters represent mean differences where p=<0.05.

Figure 3: Phosphate Transporters

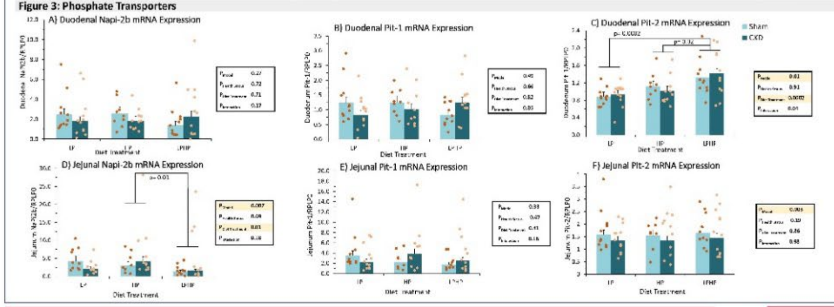


Figure 3. Phosphate Transporters: A&B) Duodenal Napi-2b and duodenal PiT1 mRNA expression was no different between any of the groups (P_{Model} = 0.27 and 0.49). **C)** However, Duodenal PiT2 was significantly higher in LPHP rats compared with either LP or HP rats ($P_{Diet\ Treatment}$ =0.0002). **D)** Contrarily, jejunal Napi-2b mRNA expression was significantly lower in LPHP rats compared with rats on the HP diet ($P_{Diet\ Treatment}$ =0.01). **E&F)** Jejunal PiT1 and PiT2 expression was no different between any of the groups (P_{Model} = 0.33 and 0.0003). The significant P_{Model} shown for jejunal PiT2 expression is due to a cohort effect. However, it is likely only statistically significant and not biologically significant.

Taken together with our previous findings, lower intestinal P absorption in response to an acute high P diet following a week of low P diet is accompanied by higher iFGF23 and iPTH as compared to a week of low P diet, and lower expression of jejunal Napi-2b mRNA as compared to a week of high P diet, yet this is not sufficient to prevent elevated plasma P.

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Schmitz, KM, et al. American Society for Bone and Mineral Research Nat'l Mtg, Seattle, WA, Sept 11-15, 2020. ePoster #P-742.

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Contact Information:
Kendal Schmitz, RDN | PhD Student – Department of Food Science and Nutrition, University of Minnesota
schm3852@umn.edu

Appendix E.6: Schmitz, KM; Cladis, DP; Vorland, C; Dong, Y; Wastney, M; Moe, S; Hill Gallant KM. 2020. Effects of Low and High Dietary Phosphorus and Acute High Dietary Phosphorus on Intestinal Phosphorus Fractional Absorption in Nephrectomized Male Rats. American Society for Bone and Mineral Research Nat'l Mtg, Seattle, WA, Sept 11-15. ePoster #P-742.

Dietary phosphorus (P) restriction and phosphate binder medications are common therapies for patients with chronic kidney disease (CKD). The goal of these therapies is to reduce intestinal P absorption to slow the development of CKD-mineral and bone disorder. However, bouts of non-adherence to these interventions could cause unintended consequences of spikes in P absorption and serum P due to increased intestinal P absorption efficiency. This has been observed in healthy rats using *in vitro* methods for assessing intestinal P uptake but has not been evaluated in CKD rats nor with *in vivo* absorption testing methods.

This study aimed to determine the effects of dietary P level in CKD and normal rats using a 2x3 factorial design. Male Sprague-Dawley rats with 5/6 nephrectomy (n=36) or sham operation (n=36) were block randomized to one of three diets: low P (LP, 0.1%), high P (HP, 1.2%), or low P followed by acute high P (LPHP, 0.1% then 1.2%) (n=12/group) and were fed for 4h/d for 7 days. LPHP rats were fed the LP diet on days 1-6 and the HP diet on day 7. Following the final feeding period, rats underwent an *in vivo* intestinal P absorption test using ^{33}P radioisotope administered either by oral gavage or IV. Fractional P absorption was determined from the ratio of area under the oral and IV plasma ^{33}P curves

(AUC_{PO}/AUC_{IV}) from serial blood draws over 2 hours. A linear mixed model was used to determine main effects of diet and disease and the interaction.

Results show a main effect of diet (p=0.03), no effect of disease (p=0.31), and no interaction effect (p=0.98). LP rats had higher fractional P absorption (0.13 ± 0.01) compared to HP rats (0.08 ± 0.01, p(diff)=0.046) or LPHP rats (0.08 ± 0.01, p(diff)=0.049). Fractional P absorption was not different between HP and LPHP (p(diff)=0.999). These results are consistent with prior studies using *in vitro* methods showing an increase in P uptake efficiency with P restriction. The lack of statistical difference for P absorption efficiency between CKD and sham rats aligns with other emerging reports that CKD does not reduce P absorption efficiency. Importantly, these results show a rapid response to reduce intestinal P absorption efficiency when an acute high P load is given following a week of dietary P restriction. This is evidence against the notion the dietary P restriction causes an unintended harm of increased absorption efficiency during dietary non-adherence.

ASBMR 2020 Annual Meeting

September 11–15, 2020

Virtual Event

Effects of Low and High Dietary Phosphorus and Acute High Dietary Phosphorus on Intestinal Phosphorus Fractional Absorption in Nephrectomized Male Rats

K.M. Schmitz,[1] D.P. Cladis,[1] C.J. Vorland,[3] Y. Dong,[2] M.E. Wastney,[2] S.M. Moe,[4] K.M. Hill Gallant[1]

[1]Department of Nutrition Science, Purdue University, Department of Food Science and Nutrition, University of Minnesota
[2]Department of Nutrition Science, Purdue University
[3]Department of Applied Health Science, Indiana University School of Public Health-Bloomington
[4]Department of Medicine-Division of Nephrology, Indiana University School of Medicine

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INTRODUCTION

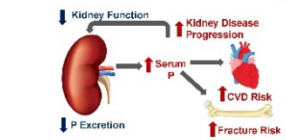


Figure 1. Dietary Phosphorus can have Negative Health Consequences for Chronic Kidney Disease (CKD) Patients. When kidney function declines so does the kidney's ability to excrete phosphorus. This leads to an increase in serum P, ultimately leading to an increase in fracture risk, CVD risk, and expedites kidney disease progression.

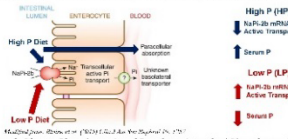


Figure 2. Dietary Phosphorus Level Regulates Intestinal Phosphorus Absorption Efficiency. P is absorbed via a regulated transcellular route and a concentration dependent paracellular route. LP diets stimulate the transcellular route through the upregulation of NaPi-2b levels, while HP diets suppress NaPi-2b and transcellular P absorption occurs primarily via the paracellular route.

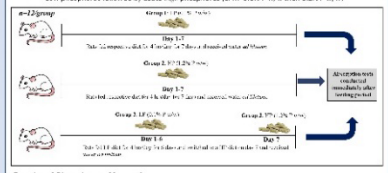
Current recommendations for CKD patients are to follow a LP diet or take P binders because of the implications a HP diet can have on serum P, kidney function, bone, and CVD risk.

However, bouts of non-adherence to the diet are common and because LP diets increase P absorption efficiency it is unknown how a bout of non-adherence affects P absorption and serum P levels

METHODS

Design:

- This study was a 2x3 factorial design in which 7-8 week old male Sprague-Dawley (CD) rats underwent a 2-step 5/6th nephrectomy (n=36) or sham operation (n=36) at Charles River Laboratories.
- After a 1-week recovery period, rats were shipped to Purdue University and fed a standard rodent chow for 3 weeks. At 4 weeks post surgery, rats were switched to a randomly assigned diet (block randomized by time) treatment of either:
  - Low phosphorus (LP 0.1% P w/w)
  - High phosphorus (HP 1.2% P w/w)
  - Low phosphorus followed by acute IVeit phosphorus (LPHP 0.1% P w/w then 1.2% P w/w)



Fractional Phosphorus Absorption:

- Fractional P absorption was determined by *in vivo* oral gavage absorption tests.
- Following the four-hour feeding period on the 7th day, 8 rats/group were orogastric gavaged with 3 mL of a transport solution enriched with 2.0 μCi ^{33}P.
  - Transport solution consisted of Na_2HPO_4 and PBS standardized to contain 6.4 mg P for rats on the LP diet treatment and 76.8 mg P for rats on the HP diet treatment.
- Four rats/treatment group underwent an IV administration of 1 mL sterile saline enriched with 5 μCi ^{32}P via an inserted jugular catheter.
  - These rats were also given an orogastric gavage of the same oral transport solution without the radioisotope at the same time as the IV ^{32}P dose.
- Blood (0.25 mL/sampling) was collected at 0, 20, 40, 60, 90, and 120 minutes after dosing with ^{32}P and was centrifuged for plasma.
  - 100 uL of plasma was placed in 15 mL of Ecolite liquid scintillation cocktail.
- Fractional P absorption was determined from the ratio of area under the oral and IV plasma ^{32}P curves (AUC_{po}/AUC_{iv}) from the serial blood draws over 2 hours.

Plasma Biochemistries:

- Baseline (start of study diet) and sacrifice blood draws were taken and centrifuged for plasma.
- Plasma was analyzed for BUN to confirm health status of rats.

Statistics:

- A linear mixed model was used to determine main effects of diet and disease and the interaction.
- Outliers in BUN were thoroughly investigated and removed from all analysis if they were greater than 2 standard deviations from the mean. Removed rat was from group CKD LP.
- One additional outlier was removed from fractional absorption that was greater than 2 standard deviations from the mean. Removed rat was from group CKD LP.

RESULTS

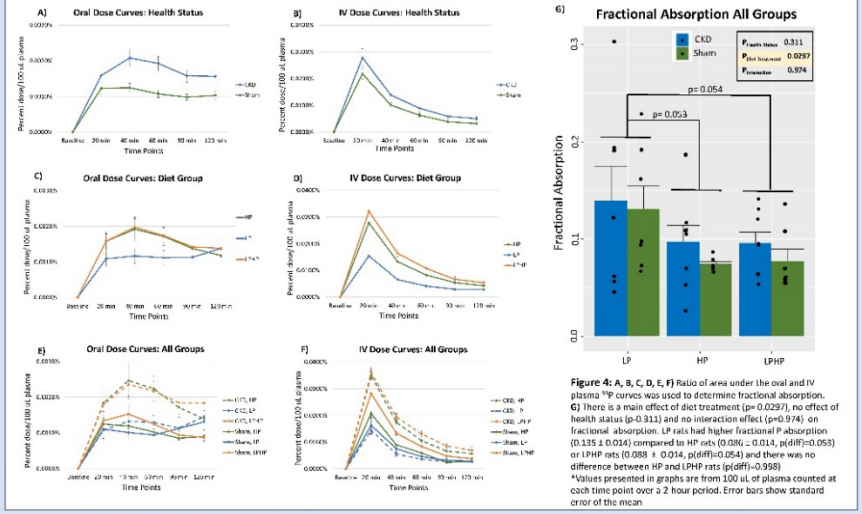


Figure 4: A, B, C, D, E, F) Ratio of area under the oral and IV plasma ^{32}P curves was used to determine fractional absorption. **G)** There is a main effect of diet treatment (p= 0.0297), no effect of health status (p-0.311) and no interaction effect (p=0.974) on fractional absorption. LP rats had higher fractional P absorption (0.135 ± 0.014) compared to HP rats (0.086 ± 0.014, p(diff)=0.053) or LPHP rats (0.088 ± 0.014, p(diff)=0.054) and there was no difference between HP and LPHP rats (p(diff)=0.998)
*Values presented in graphs are from 100 uL of plasma counted at each time point over a 2 hour period. Error bars show standard error of the mean

HYPOTHESIS

Diet effects: Fractional phosphorus absorption will be highest in rats adapted to the LP diet then acutely switched to the HP diet prior to absorption testing; followed by rats kept on the LP diet; and lowest in rats fed the HP diet.

CKD effects: Fractional phosphorus absorption will be higher in CKD rats compared with sham-operated rats based on findings of our previous study in the Cy+ model.

RESULTS

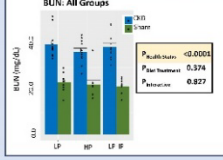


Figure 3. BUN: CKD rats had higher BUN (38.5± 1.028) compared to sham rats (22.1± 1.013), (*p=<0.0001). There was no difference between diet treatment (p-0.374) or the interaction (p-0.827). *Analysis was performed using log transformation but data are presented as back transformed values. Error bars show standard error of the mean.

CONCLUSIONS

- In contrast to our hypothesis, CKD rats did not have higher fractional P absorption compared to sham rats. Instead, there was no difference in fractional P absorption between CKD and sham rats.
- Fractional P absorption was influenced by P load, which is consistent with prior studies using *in vitro* methods showing an increase in P uptake efficiency with P restriction.
- There was a rapid response to reduce intestinal P absorption efficiency when an acute high P load was given following a week of dietary P restriction.

These data provide evidence against the notion that dietary P restriction causes an unintended harm of increased absorption efficiency during dietary non-adherence.

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