CONTROLLED DIET STUDIES OF INTESTINAL PHOSPHORUS ABSORPTION IN CHRONIC KIDNEY DISEASE

by

Elizabeth R. Stremke

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Nutrition Science

West Lafayette, Indiana

May, 2020

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Kathleen Hill Gallant, Chair

Department of Nutrition Science, Purdue University

Dr. Sharon Moe

Division of Nephrology, Department of Medicine, Indiana University School of Medicine

Dr. Regan Bailey

Department of Nutrition Science, Purdue University

Dr. Laura Bowers

Department of Nutrition Science, Purdue University

Approved by:

Dr. Kimberly K. Buhman

Head of the Graduate Program

To my person, Ryan M. Stremke.

The truest partner I could ever hope for. I love you, always.

To Jansen Michael and Margaret Joy

The lights of my life and my motivation. I love you, always.

ACKNOWLEDGMENTS

To my mentor, Dr. Katie Hill Gallant: What do I say that could properly acknowledge you? You have been an incredible mentor for me in every aspect of my life; your support during each season of my academic, and personal, life has been immeasurable. You are an amazing scientist, mentor, and woman. I could fill this entire page acknowledging you and how you have literally come to my rescue countless times. Instead, I will say it has been one of my greatest honors to be your student. I am the person that I am today because I met and worked with you. Your friendship has meant more to me than you can ever know and I can't wait to mentor my own students one day, because of you.

Thank you to my committee members: Dr. Sharon Moe, Dr. Regan Bailey, and Dr. Laura Bowers. Your guidance and confidence in me has helped me move through this program and has prepared me to take my next steps forward. Thank you to all of my INP friends and lab mates who have been on this journey with me. Meg, Andrea, Gretchen, Maria, Mike, Julia, Kendal, Violet, Dennis, Heng, Regina, Krittikan, Chae, and Wei: you are all wonderful.

To Dr. Colby Vorland: I wouldn't have made it through without you, buddy. Thank you for always being there when I needed to talk, for your patience when I had questions, and for just generally making me feel less crazy during crazy times. Oh yes, and thank you for always eating my leftover food. ☺

To my very first female scientist role model: Dr. Carin Vanderpool. I remember looking at your bound dissertation when I was little and thinking "Wow. Cari wrote an entire BOOK." Thank you for paving the way for me, and all of our family. You are an amazing big sister and role model for everyone around you.

To my entire family (Vanderpool and Stremke): Thank you for all of the support and understanding you have given us over the years. Because of your help, my kids got to watch me complete a huge goal and understand the importance of education and curiosity. Your help has not been taken for granted.

Lastly, to my partner. Ryan. These works are dedicated to you and our children. Each day I count my lucky stars to have what we have. Every ounce of effort put into this has been for you and for them. I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Chronic kidney disease (CKD) affects approximately 37 million American adults with many more at risk for disease development. Elevated serum phosphorus and related abnormalities in phosphorus homeostasis due to progressive loss of kidney function are primary driving forces behind cardiovascular dysfunction and mortality in CKD patients. Intestinal phosphorus absorption is an understudied aspect in phosphorus homeostasis. Despite this lack of information, current therapies focus on reducing intestinal phosphorus absorption via the use of oral phosphate binders and dietary phosphorus restriction. Abnormalities in phosphorus-regulating hormones are already present in early stages of CKD, including elevated FGF23 and decreased 1,25-dihydroxyvitamin D, which should have the effect of reduced phosphorus absorption. However, rodent studies suggest that intestinal phosphorus absorption remains at inappropriately normal levels in CKD, despite perturbations in phosphorus homeostatic hormones. In these works, we studied intestinal phosphorus absorption in patients with CKD, with a particular emphasis on controlled diet studies, and on method of intestinal phosphorus absorption assessment.

In our first study, we assessed the variation and reliability of 24-hour urinary phosphorus excretion, a presumed biomarker of intestinal phosphorus absorption, in N=8 moderate-stage CKD patients. Here we found a high degree of variability in 13-consecutive measures of 24-hour urine phosphorus measures in patients consuming a controlled diet. We also found that at least two measures of 24-hour urine phosphorus is required for a reliable measure. Lastly, we found that in these moderate CKD subjects, 24-hour urine phosphorus is not related to net phosphorus absorption. However, this does not preclude the existence of a relationship in interventional studies assessing direct methods of intestinal phosphorus absorption suppression (i.e. efficacy studies of phosphorus binders) and urinary phosphorus excretion.

Next, we assessed fractional intestinal phosphorus absorption in N=8 moderate-stage CKD patients compared to N=8 healthy adults matched for age, sex, and race. In this study, we administered the radioisotopic tracer, 33-Phosphorus, via oral and intravenous routes, staggered by exactly 25-hours. This mimics the gold-standard method of dual-administration of two different isotopes. In our study, the use of a single isotope is a safer method as 33-Phosphorus is lower energy compared to 32-Phosphorus, a higher energy isotope used in some previous studies of end-stage kidney disease. Our results showed that fractional intestinal phosphorus absorption was

similar between CKD patients and healthy adults, as we hypothesized. These findings were despite significantly lower values of serum 1,25D in CKD patients compared to healthy adults.

Lastly, we considered the effect of adherence to a low-phosphorus diet on serum phosphorus area-under-the-curve during the intradialytic period in hemodialysis patients. In this secondary analysis of a placebo-arm of a drug trial that included a controlled diet, we examined the post-dialytic serum phosphorus rebound in N=13 hemodialysis patients. We found that, compared to previous reports in the literature, adhering to a low-phosphorus diet in hemodialysis patients may delay the serum phosphorus rebound in the intradialytic period. In our study, only 2 of 13 patients had returned to their pre-dialysis serum phosphorus values at 24-hours post-dialysis and 4 of 13 at 48-hours. Importantly, all patients were not using phosphate binder medications for the duration of the study. These data show a potential benefit of adherence to a low phosphorus diet for phosphorus control, even in the absence of phosphate binder medications. However, longer studies of a controlled low phosphorus diet compared with a normal phosphorus diet on serum phosphorus rebound in the intradialytic period is needed to substantiate these findings.

The results from our studies are foundational in the understanding phosphorus absorption in moderate CKD. This knowledge will be critical for the development of translational interventions to limit phosphorus burden at earlier stages of the disease, thus limiting additional risk for disease progression and mortality.

CHAPTER 1: INTRODUCTION

Portions of this review have been published elsewhere, listed here:
Stremke, E.R., Hill Gallant, K.M. Intestinal Phosphorus Absorption in Chronic Kidney Disease. *Nutrients,* 2018. 10(10).

Chronic kidney disease (CKD) affects approximately 37 million American adults in the United States and approximately 1 in 3 people are at risk (1). CKD is a disease of progressive decline in renal function that includes disruption of normal phosphorus homeostasis as the kidney loses its ability to regulate phosphorus by urinary excretion. Notably, abnormalities in phosphorus homeostasis have been shown to occur early in CKD progression due to disease-mediated alterations in the hormonal regulators of phosphorus metabolism, well before clinical hyperphosphatemia is observed (2). This is a central component of CKD-mineral bone disorder (CKD-MBD), which is a condition characterized by 1) abnormalities in laboratory values related to calcium and phosphorus metabolism, 2) vascular calcifications, and 3) bone disease, all of which interact and contribute to increased risk for cardiovascular events, bone fragility fractures, and death (3). In fact, the leading cause of death in patients with CKD is cardiovascular disease, not kidney failure (4). Higher serum phosphorus itself has been associated with disease progression in CKD patients (5). Because disturbed phosphorus metabolism is considered an instigating factor in the development and progression of CKD-MBD, current therapies for CKD-MBD include controlling phosphorus abnormalities. Much of this is aimed at limiting absolute intestinal phosphorus absorption since the failing kidney is not, at present, a suitable point of intervention.

Hormonal Control of Phosphorus and its Dysregulation

Phosphorus homeostasis is controlled by a multi-tissue axis that includes the kidney, intestine, bone, and parathyroid glands (6) as shown in Figure 1.1A. Dietary phosphorus is absorbed via the intestine, while the bone is the major reservoir for the body's phosphorus stores, and the kidney filters, reabsorbs, and excretes phosphorus from the body. The parathyroid glands, bone, and kidney are also endocrine organs that produce the main known phosphorus regulating hormones: parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1,25-dihyxdroxyvitamin D (1,25D), respectively. These hormones have complex functions to regulate

phosphorus homeostasis, but also regulate one another in a series of negative feedback loops (7). Figure 1.1B is a graphical depiction of these feedback loops and Figure 1.2 is a detailed diagram of hormonal control of phosphorus by Bergwitz and colleagues (7).

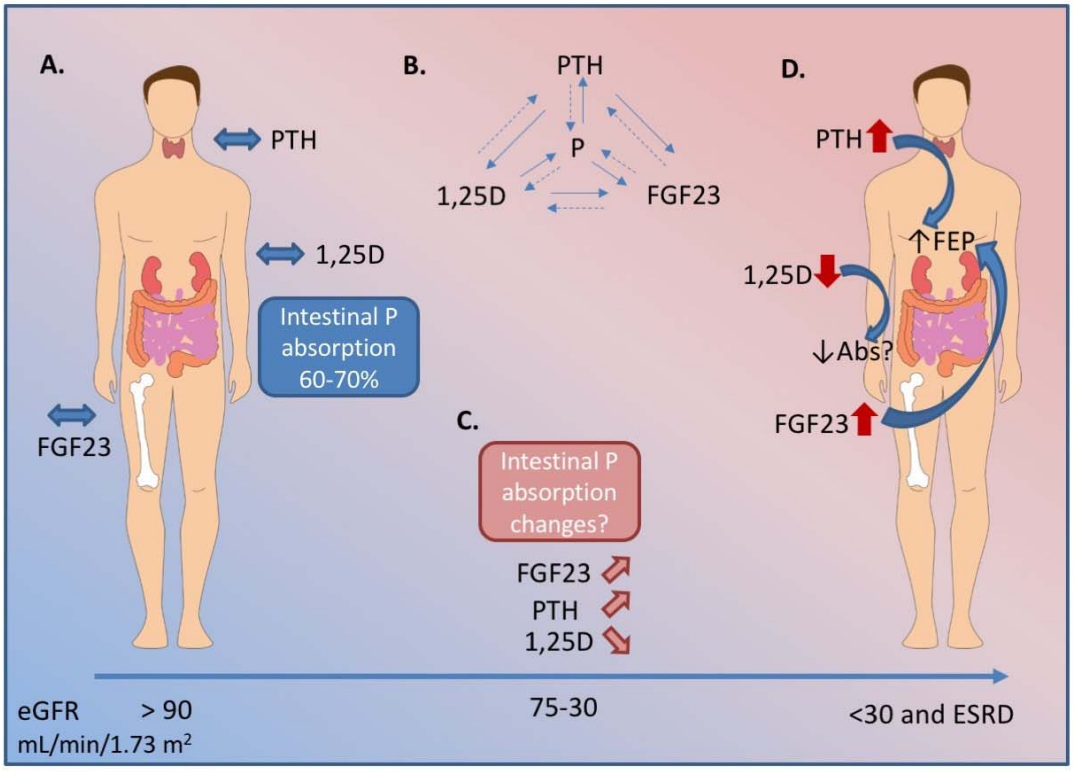


Figure 1.1 Biochemical changes in hormonal regulators of phosphorus throughout the course of CKD. **A.** In health, FGF23 from osteocytes, PTH from the parathyroid gland, and 1,25D produced in the kidney work together to keep serum phosphorus within a normal range. A healthy person absorbs approximately 60-70% of P in a mixed diet. **B.** PTH, 1,25D, and FGF23 also regulate each other in a series of feedback loops. **C.** As renal decline progresses, the kidney loses its ability to excrete excess P. FGF23 begins to rise earlier in the disease course, followed by a decline in 1,25D and a rise in PTH. **D.** In end stage renal disease, the hormonal regulation is unable to maintain serum phosphorus within a normal range. At this stage, patients have very high FGF23, PTH, and low 1,25D. This biochemical profile in early and moderate stage CKD indicate intestinal P absorption should be decreased. However, some prior literature suggests P absorption may be inappropriately maintained and therefore contributing to overall P burden in CKD. Key: Dashed lines indicate suppressive effects and solid lines indicate stimulatory effects in the negative feedback loops. "Abs" indicates intestinal P absorption; "FEP" indicates fractional excretion of P from the kidney. *Reprinted with permission from Nutrients.*

Phosphorus is distributed in different physiological compartments in the body. In a healthy individual, there is about 700 grams of total phosphorus in the body. Eighty-five percent (85%) of the phosphorus in the body resides in the skeleton in the form of hydroxyapatite. Intracellular spaces house 14% of phosphorus, while only 1% of whole body phosphorus is represented in blood and extracellular fluid (ECF) (8). Of this 1%, 70% of phosphorus in the ECF is present as organic phospholipids and 30% as inorganic phosphorus. Further, 15% of the inorganic phosphorus is bound by proteins leaving 85% of the inorganic phosphorus fraction (a miniscule amount compared to whole-body phosphorus stores) of the ECF to be measured by laboratory assays for blood biochemistries (8, 9).

To the best of current knowledge, the primary signal to initiate phosphorus homeostatic mechanisms is an alteration of ECF, or serum, phosphorus. Indeed, understanding the complex relationship of these hormones is best understood through the lens of these alterations; however mechanisms of phosphate-sensing are poorly understood. In response to increased serum phosphorus, the parathyroid glands provide a rapid response by increasing PTH secretion. While PTH primarily acts to maintain calcium homeostasis (10), PTH is also released in response to elevated serum phosphorus (11 ,7,12). PTH is a phosphaturic hormone that decreases renal phosphorus reabsorption (6), thus increasing urinary phosphorus excretion. PTH also stimulates the renal conversion of 25-hydroxyvitamin D to 1,25D via the CYP27B1 enzyme. The main role of 1,25D in phosphorus homeostasis is to increase intestinal phosphorus fractional absorption (13-15). Both PTH and 1,25D stimulate production of the third main, and most recently discovered, phosphorus-regulating hormone: FGF23. FGF23 is produced by osteocytes in bone (7,12-16). Like PTH, FGF23 is a phosphaturic hormone that regulates serum phosphorus. The main function of this hormone is to promote phosphaturia (12). This is evidenced by patients who have genetic disorders that cause extremely high serum intact FGF23 levels. These patients exhibit severe renal phosphorus wasting, hypophosphatemia, and rickets (17). FGF23 and PTH both promote phosphaturia by decreasing expression of phosphorus transporters in the brush border membrane of the proximal convoluted tubule in the nephron (7,18). However, the decrease in renal phosphorus reabsorption stimulated by FGF23 is independent of PTH action (19) as FGF23 also inhibits PTH (18). It is important to note that although both PTH and FGF23 promote phosphaturia, FGF23 is a stronger regulator of phosphorus excretion than PTH (18). When the rapid release of PTH is not sufficient, FGF23 secretion is stimulated by sustained serum phosphorus elevations to

cause a strong phosphaturic response. Negative feedback loops between PTH, 1,25D, and FGF23 exist and are critical to regulate these hormones' control over phosphorus homeostasis. While PTH and 1,25D increase FGF23, FGF23 decreases PTH synthesis and secretion as well as decreases renal production of 1,25D (10, 7) PTH stimulates 1,25D production, and 1,25D in turn downregulates itself and PTH (7, 18-20). These hormones alter phosphorus homeostasis through the regulation of phosphorus transporters located in the brush border membrane of the renal and intestinal epithelial cells.

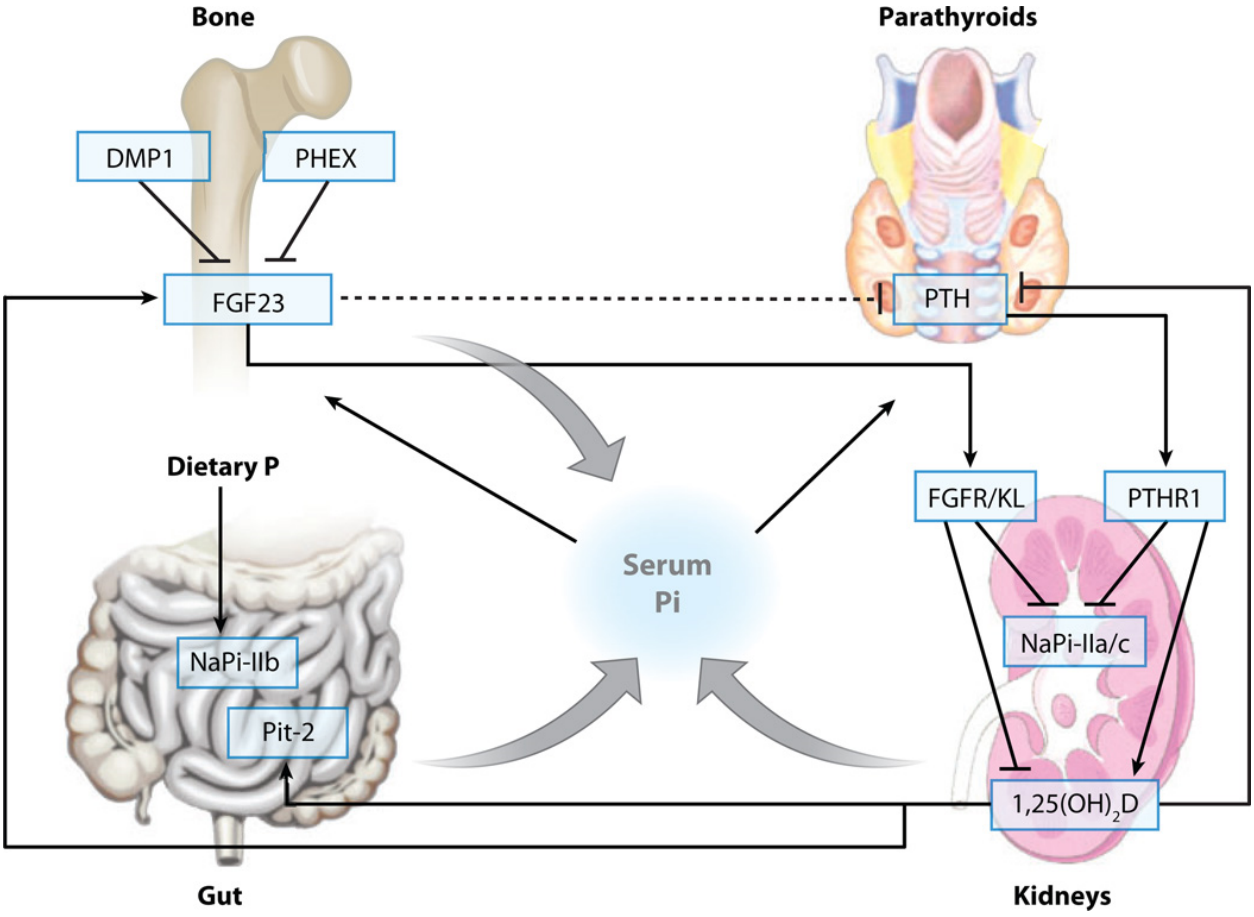


Figure 1.2 Hormonal control of phosphorus in a multi-tissue axis. *Reprinted with permission from Annual Review of Medicine (7).*

Because the kidney is the main regulator of phosphorus homeostasis, the decline in kidney function that occurs in CKD affects phosphorus homeostasis. CKD is a progressive disease that is categorized into stages based on estimated glomerular filtration rate (eGFR) ranging from stage 1 (mild with normal to increased eGFR) to stage 5 (severe with < 15% eGFR). Alterations in phosphorus homeostasis begin to occur in the early stages of the disease and are able to maintain

serum phosphorus in a normal range until late CKD (2). Data from Chronic Renal Insufficiency Cohort, shown in figure 1.3, show that the first alteration observed as renal function declines is elevated serum FGF23, followed be decreased serum 1,25D, then elevated serum PTH, and eventually elevated serum phosphorus. It is important to note that serum phosphorus levels are maintained within normal range by the actions of FGF23, PTH, and 1,25D until late CKD, when renal function is so severely impaired that these hormonal compensations are inadequate (18, 21, 22). Some have proposed that the early rise in FGF23 may be caused by a deficiency of the FGFR membrane co-receptor, klotho (2, 18, 19). Klotho concentration declines with age and with CKD progression (23-25). Klotho deficiency leads to FGF23 resistance and continual secretion of FGF23 from osteocytes (18). It is proposed that FGF23 might then develop affinities for other FGFRs that do not require klotho as a co-receptor (i.e. FGFR4 in the heart) and stimulate adverse effects in off-target tissues (18, 26). However, elevated serum phosphorus is associated with vascular calcifications and death in patients with CKD (27). So, these hormonal alterations are necessary to control serum phosphorus. But, these alterations do not occur without consequence. For example, elevated FGF23 has been shown to directly induce left ventricular hypertrophy in mice, which supports observations of greater left ventricular mass with higher levels of FGF23 in patients with CKD (26).

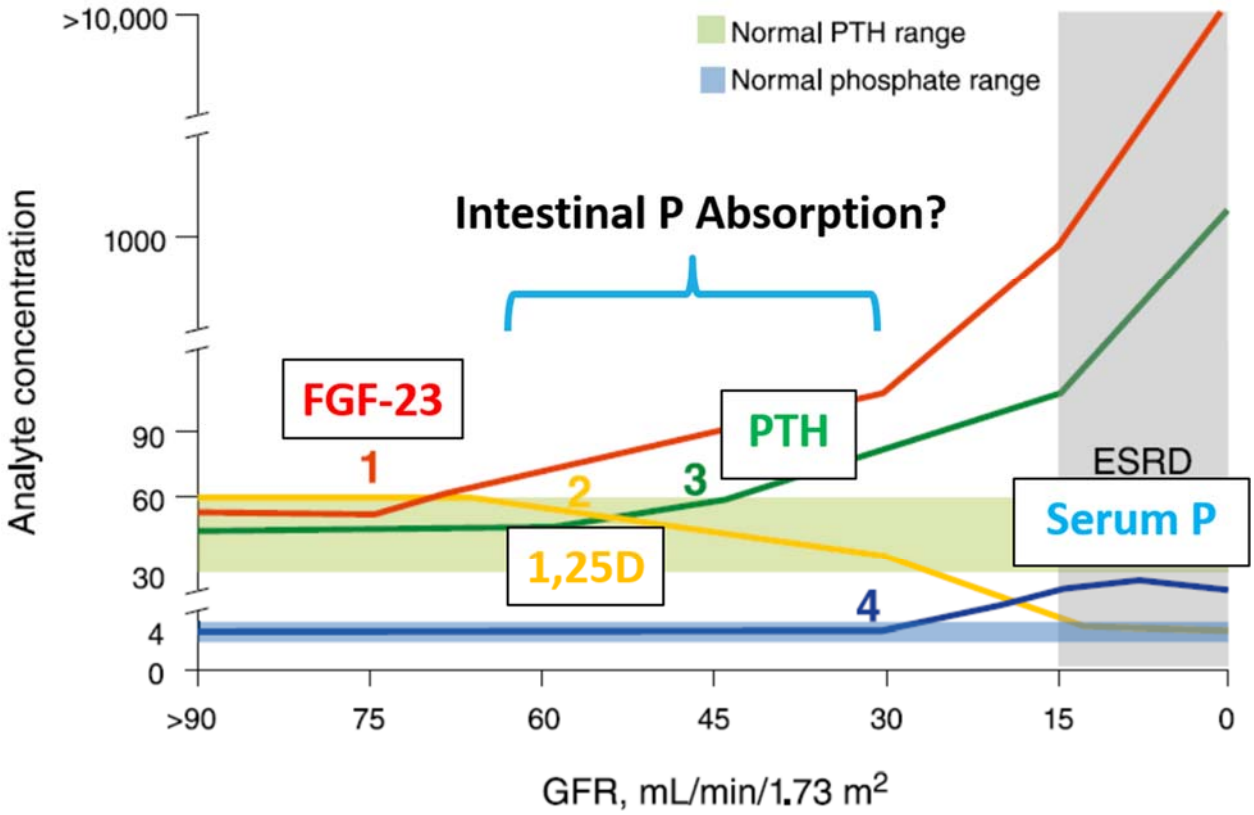


Figure 1.3 Changes in phosphorus hormonal control as eGFR declines.
Adapted from Wolf, 2015. *Reprinted with permission from Clinical Journal of the American Society of Nephrology (22).*

Mechanisms of Renal Phosphorus Excretion

What is known about phosphorus absorption was preceded by the discovery of active phosphorus transport in the renal proximal tubules (28). In the kidneys, 85% of phosphorus is reabsorbed along the proximal convoluted via a transcellular route. Smaller proportions of phosphorus are also reabsorbed along the Loop of Henle (10%), distal convoluted tubule (3%) and collecting duct (2%) by unknown transport mechanisms (29). Figure 1.4. shows the localization and movement of phosphorus through transporters in the proximal convoluted tubule. Phosphorus is cotransported via sodium-dependent cotransporters NaPiIIa, NaPiIIc, and PiT2. NaPiIIa transports divalent forms of phosphorus (HPO_4^{2-}) in a 3:1 ratio sodium:phosphorus making the transport process electrogenic. NaPiIIc, however, transports divalent forms of phosphorus on a 2:1 ratio making it electroneutral. PiT2 transports monovalent forms of phosphorus ($H_2PO_4^-$) on a 2:1 ratio and is also electroneutral (30).

PTH and FGF23 directly impact the stabilization of NaPi proteins in the membrane. NaPiIIa is stabilized in the brush border membrane by a protein called NHERF1 while NaPiIIc is largely stabilized by PDZK1. Upon PTH stimulation of PTHR1 and PTHRP, the PKA/PKC signaling pathway phosphorylates NHERF1. This phosphorylation causes the protein to dissociate from NaPiIIa and allows NaPiIIa to be taken out of the membrane via endocytosis and degraded. Similarly, FGF23 stimulation acts to phosphorylate NHERF1, but through the ERK1/2 pathway (30).

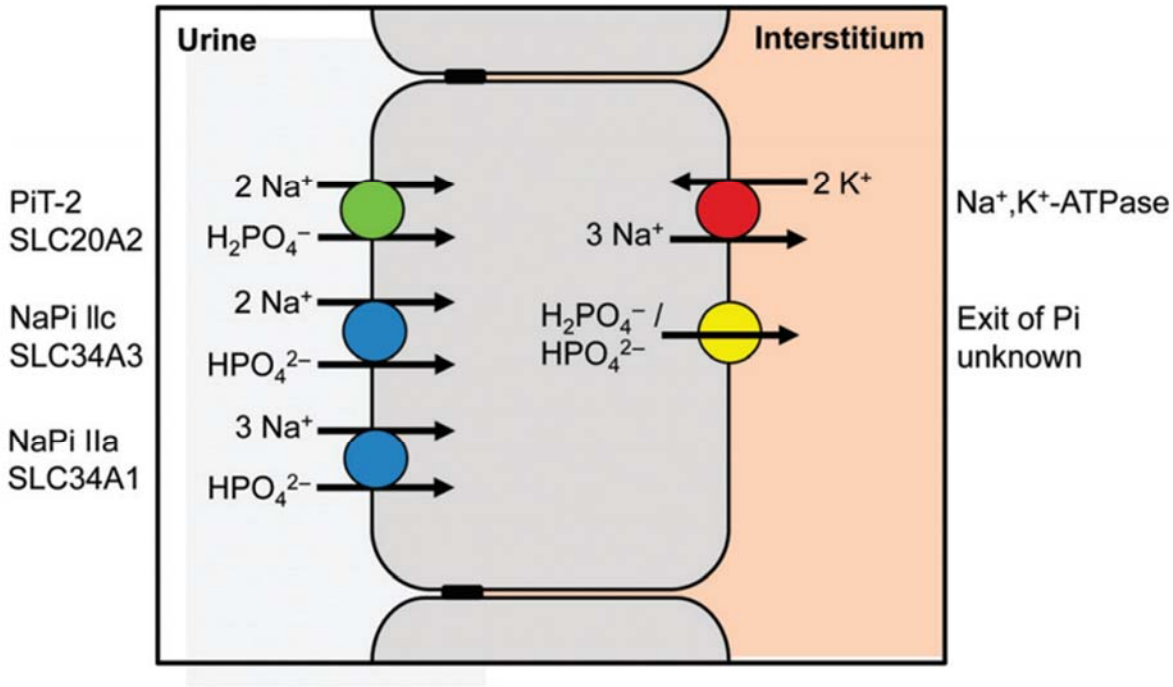


Figure 1.4 Mechanisms of Renal Phosphorus Transport in the Proximal Convoluted Tubule.
Reprinted with permission from Comprehensive Physiology (31).

Mechanisms of Intestinal Phosphorus Absorption

Intestinal phosphorus absorption occurs similarly to renal phosphorus reabsorption in that, like the kidney, the intestine contains an active, sodium- dependent component of phosphorus absorption. The intestine expresses both type II and type III sodium-dependent phosphate transporters and occurs via sodium-dependent (transcellular) pathway (32). NaPiIIb, analogous to renal transporter NaPiIIa, is the lone type II transporter expressed in the intestine and is encoded by the gene SLC34A2 (32). NaPiIIb has the highest affinity for divalent phosphate (HPO_4^{-2}), and

transports sodium and phosphorus in a 3:1 ratio across the membrane. This transport is made possible by the efflux of sodium on the basolateral side of the cell, leaving a lower concentration of sodium intracellularly (33). Transcellular transport ultimately produces a net positive charge in the intracellular space. Type III, sodium-dependent, phosphorus transporters PiT-1 and PiT-2 are expressed in the human intestine (32). PiT transporters cotransport monovalent phosphate ($H_2PO_4^-$[1]) in a 2:1 ratio of sodium:phosphate. Although studies have suggested that type III transporters may compensate for intestinal phosphorus absorption in times where type II transporters are compromised (32), *in vitro* studies of phosphorus absorption performed on murine ileum segments have shown that that NaPiIIb is responsible for a majority (reported >90%) of transcellular phosphorus transport (34, 35), but that transcellular transport accounts for only 50% of total phosphorus transport in the intestine (34).

The intestine also transports phosphorus in a sodium independent, paracellular manner (also referred to as passive absorption). This is in contrast to the kidney where phosphorus transport is completely negated when sodium is not present in the tubules (36). This indicates that no paracellular phosphorus transport occurs in the renal tubules. Paracellular phosphate transport is unsaturable and is directly related to the phosphorus load in the intestinal lumen (37, 14) and is traditionally considered unregulated. However, regulation may yet exist and may particularly involve tight junction proteins such as claudins (32), but this is an emerging and understudied area. Figure 1.5 is a visualization of intestinal phosphorus absorption across an intestinal epithelial cell. The left side demonstrates proportion of absorption through the paracellular and transcellular pathway on a high phosphorus diet. The right demonstrates absorption via these pathways on a phosphorus restricted diet. There are several known regulators of transcellular sodium-dependent phosphate transport in the intestine, namely 1,25D and dietary phosphorus intake.

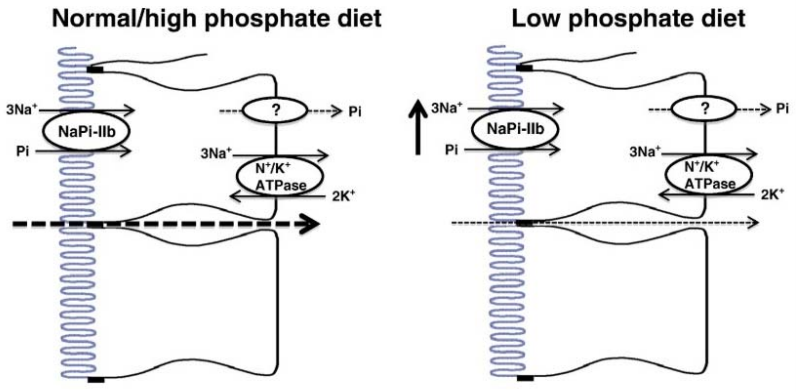


Figure 1.5 Intestinal Phosphorus Absorption Across the Intestinal Epithelial Cell. *Reprinted with permission from Pflugers Archiv : European journal of physiology (38).*

Role of 1,25-Dihydroxyvitamin D in Regulation of Intestinal Phosphorus Absorption

The role of 1,25D in the regulation of intestinal fractional phosphorus absorption is well established. Early studies have shown healthy rats and rats replete with vitamin D have increased phosphate flux and phosphate uptake across the intestinal brush border membrane (13, 39). Specifically, Walling (13) showed that 1,25D repletion of vitamin D deficient rats increased active transport of phosphorus across all segments of the intestine. In accordance with increased phosphorus flux, 1,25D treatment increases gene expression of the type III transporter PiT-2 (39). In contrast to type III transporters, evidence supports that NaPiIIb expression is post-transcriptionally regulated by 1,25D. Hattenhaur et al. also found that treatment with cholecalciferol did not change NaPiIIb transcript, but did increase NaPiIIb Vmax. These data are consistent with Walling (13) and Katai (39) and suggest that 1,25D influences the amount of NaPiIIb protein located in the intestinal brush border membrane where it is active. More evidence to support this non-genomic role of 1,25D in the regulation NaPiIIb is that NaPiIIb does not have a vitamin D response element (VDRE) in its genome (40). Other studies have suggested that perhaps 1,25D has non-genomic control over intestinal phosphorus absorption, but these studies have not linked the non-genomic action with intracellular signaling pathways that control the NaPiIIb protein (41). Interestingly, the effect of dietary phosphorus restriction on intestinal fractional phosphorus absorption was previously thought to be a result of decreased systemic 1,25D. However, recent studies have shown that dietary phosphorus restriction has a vitamin D-

independent effect on intestinal fractional phosphorus absorption (42). Details on the physiologic impact of dietary phosphorus load are discussed in the following section.

Role of Dietary phosphorus Load in Intestinal Phosphorus Absorption

Dietary phosphorus restriction is another factor known to affect both intestinal fractional phosphorus absorption and absolute phosphorus absorption. Because passive phosphorus absorption is dependent on the intestinal luminal phosphorus load, absolute phosphorus absorption is greater with higher levels of dietary phosphorus and is lower with lower levels of dietary phosphorus, as described above. The opposite relationship is observed between dietary phosphorus intake level and intestinal fractional phosphorus absorption (i.e. absorption efficiency). An early observation of this was reported by Lee et al. (43). Young rats were fed a phosphorus restricted diet of 0.03% phosphorus (~1/10 of their dietary phosphorus requirement) for six weeks which resulted in increased jejunal fractional phosphorus absorption. However, this study represents severe dietary phosphorus restriction and therefore these data may not be translatable to "normal" physiology. But, others have since shown that even modest dietary phosphorus restrictions elicit similar responses in phosphorus fractional absorption. Giral et al. (44) showed increased phosphorus uptake into brush border membrane vesicles isolated from the jejunum in young male rats fed a low phosphorus diet (0.1%, ~1/3 of the dietary requirement) compared to higher phosphorus diets (0.6% & 1.0%). Saddoris et al. (45) also showed increased intestinal phosphorus uptake in weanling pigs fed a diet moderately (43%) reduced in phosphorus.

Because of the established effect 1,25D on increasing fractional phosphorus absorption, and because low phosphorus diets cause an elevation in 1,25D (46, 47), it was thought that low phosphorus diets affected fractional phosphorus absorption via a 1,25D mechanism. However, studies in vitamin D receptor (VDR) knockout and CYP27B1 knockout mice have demonstrated that the effect of low phosphorus diets on intestinal phosphate transport is vitamin D-independent. For example, Capuano et al. (42) examined the effect of a 5-day low phosphorus (0.1%) diet on intestinal NaPiIIb mRNA and protein levels in male VDR -/-, CY27B1 -/-, and wild type 10-12 week old mice. Results showed that the dietary induced changes were similar in wild type mice, VDR -/- and CYP27B1 -/- mice. The low phosphorus diet increased NaPiIIb gene expression between 3 and 5 fold in all mice regardless of genotype (42). Similarly, Segawa et al. (48) studied 8-week-old VDR (-/-) mice and found that a low phosphorus diet (0.25%) for 4 weeks induced a

greater than two-fold upregulation of NaPiIIb mRNA that corresponded with a one-fold increase in NaPiIIb protein expression. Collectively, these data indicate that intestinal regulation of NaPiIIb and intestinal fractional phosphorus absorption by dietary phosphorus restriction is not dependent on the genomic actions of 1,25D through VDR.

Clinical Strategies Employed to Control Serum phosphorus in CKD

A major strategy in the clinical management of CKD-MBD is to decrease phosphorus load by impairing intestinal phosphorus absorption. This includes use of dietary phosphorus restriction as well as phosphate binder medications. A combination of these two therapies is often required to for phosphorus control in CKD, but also becomes insufficient as renal function continues to decline (9). A dietary phosphorus restriction of 800-1000 mg/day is recommended to CKD patients to maintain serum phosphorus and serum PTH within expected values to prevent secondary hyperparathyroidism, a common comorbidity in CKD-MBD (49).

Dietary phosphorus restriction is not easily achieved or maintained. Adherence is difficult for patients because of the difficult balance between achieving adequate protein and low phosphorus, particularly in dialysis patients who have high protein needs. Phosphorus is contained in many naturally occurring forms in the American diet as well as in many food additives (50, 51). Thus, actual intake of phosphorus in CKD patients is often higher than the KDOQI recommendations of 800-1000 mg/day (52, 53). Not only is the ubiquity of phosphorus in the diet problematic, but the high bioaccessibility (estimated at 90-100%) of phosphorus-containing food additives contributes to a higher phosphorus burden compared with naturally occurring forms in plant and animal foods that have an estimated bioaccessibility between 40-70% (54, 55). Because of their estimated high bioaccessibility, limiting phosphorus-containing food additives has emerged as a novel treatment strategy to reduce phosphorus burden in patients with CKD. Sullivan et al. (56) demonstrated that an educational intervention by a registered dietitian to teach hemodialysis patients how to avoid consuming phosphorus-containing food additives had a significant effect on reducing serum phosphorus over the 3-month study compared with standard of care. This was corroborated recently by de Fornasari et al. (57) who also showed a significant reduction in serum phosphorus over 3 months in adult hemodialysis patients who were randomized to a similar education intervention to reduce phosphorus-containing food additives. Importantly, this reduction in serum phosphorus was achieved without affecting markers of nutritional status.

There is also new randomized controlled trial evidence (58) that high dietary phosphorus intake from highly-bioaccessible phosphorus-containing food additives increases blood pressure in healthy adults, which may imply a role of phosphorus metabolism in the prevalent etiology of hypertension leading to CKD as well as CKD-exacerbated hypertension. The concept of utilizing knowledge of phosphorus bioaccessibility to improve phosphorus status in patients with CKD has also been applied to interventional studies of plant-based versus meat-based diets (26, 59). These studies have shown that switching from meat-based diets with higher phosphorus bioaccessibility to plant-protein based diets with lower bioaccessibility can lower serum phosphorus or reduce 24-hour urine phosphorus excretion (reflecting reduced absolute phosphorus absorption in response to a controlled intervention). These approaches, limiting phosphorus-containing food additives and/or shifting to a plant-protein based diet, are particularly of interest as they offer an opportunity to potentially reduce phosphorus burden without sacrificing protein intake (60).

Phosphate binders offer another commonly-used strategy to prevent the absorption of dietary phosphorus. Phosphate binders are considered to be the second-line therapy when dietary restriction of phosphorus is insufficient at lowering serum phosphorus (49). There are many different types of binders with varying binding capacities of phosphorus (49). These are broadly categorized as calcium-based and non-calcium-based, with a newer category of iron-based. There is ongoing debate (61, 62) regarding the use of calcium-based phosphate binders in light of observations that they may cause calcium retention (63) from calcium balance studies in CKD (63, 64). Higher calcium retention in CKD is problematic for increased vascular calcifications (65).

Compliance with phosphate binders is also a problem due to pill burden and various side effects (52). Several types of phosphate binders are available and are generally categorized as calcium-based and non-calcium-based. All phosphate binders on the market have been shown to be effective at reducing serum phosphorus at least in short-term studies (66). Several factors like the size and number of pills, gastrointestinal side-effects, and ability to chew (needed for lanthanum carbonate) must be taken into consideration for an individual patient. A recent review on phosphate binders gives a comprehensive overview and comparison of available binders (67). Overall, the binding capacity of any phosphate binder has its limits where these alone cannot fully negate phosphorus surplus by late stage CKD. Dialysis patients experience a 300-500 mg/day phosphorus surplus if consuming 900 – 1500 mg/day of phosphorus (52) while phosphate binders have a binding capacity of approximately 250 mg/day. This excess phosphorus, if absorbed, can

contribute to hyperphosphatemia (52). Thus, many CKD patients are unable to achieve targets for serum phosphorus (52). Because of inability of the kidney to sufficiently excrete phosphorus, the intestine is a logical target for therapies aimed at reducing phosphorus burden. However, a better understanding of intestinal phosphorus absorption mechanisms, factors affecting absorption, and how CKD is a modifying factor are needed to develop better strategies for phosphorus management.

The Renal Diet and Kidney Disease Progression

As mentioned in the previous section, dietary recommendations are a key aspect of patient care in CKD and ESRD. The renal diet is prescribed to patients with declining kidney function to both help prevent disease progression and comorbidity complications (68). This diet is composed of low amounts (but high-quality) dietary protein, and low phosphorus, potassium and sodium. Table 1.1. describes dietary recommendations for patients across stages of CKD (69, 68). Seminal studies in kidney failure considered decreased dietary protein intake as a strategy to delay kidney decline in animal models (70, 71). Early observations of acute kidney injury progressing to renal disease found the progression was instigated by progressively higher glomerular pressure (70). Lower dietary protein can help prevent renal decline by reducing the overall filtration pressure in the glomeruli (70, 72, 73) and decreasing net acid excretion (74). As expected given these findings, one recent study demonstrated that higher daily dietary protein intake in a cohort including all stages of CKD was associated with a higher rate of kidney decline over 6 years (75).

The Modification of Diet in Renal Disease (MDRD) study is one of the largest studies describing a large group of patients following a protein restriction in moderate CKD. While the primary study's results were negative (for further details see Klahr, 1994 (76)), secondary analyses of this study (77) found that patients who were adherent with a low-protein intake had slower eGFR decline when compared to patients consuming a normal protein diet (77). Another recent re-analysis found that the lower protein cohort of the MDRD study experienced a small reduction in serum phosphorus that was sustained over a three year period compared to a usual protein group (78). Using dietary protein as one mechanism to control phosphorus homeostasis in conjunction with delaying kidney disease progression has implications of controlling the negative impact hyperphosphatemia and associated hormones (FGF23, and PTH) have on fracture risk, cardiovascular dysfunction, and mortality in CKD.

Table 1.1 Dietary Recommendations Across Stages of CKD

CKD stage	**Energy (kcal/kg)**	**Protein (g/kg body weight/day)**	**Phosphorus (mg/day)**	**Potassium (g/day)**	**Sodium (g/day)**
CKD Stage 1-4	35 kcal/kg for patients <60 y.o. 30 kcal/kg for patients > 60 y.o. (79)	0.6-0.8 g/kg/day (79, 68)	800-1000 mg/day for patients with serum phosphorus greater than 4.6 mg/dL (80, 81)	No restriction until hyperkalemia is present (68)	< 2.3 g/day (82)
ESRD; Dialysis	35kcal/kg for patients <60y.o. 30-35 kcal/kg for ≥ 60 y.o. (79)	HD: 1.2g/kg/d PD: 1.2-1.3 g/kg/day and up to 1.5 g/kd/d in case of peritonitis (79)	800-1000 mg/day for patients with serum phosphorus greater than 4.6 mg/dL (80)	≤2-4 g/day (80, 68)	HD: 2-3 g/day PD: 2-4 g/day (68)

Targeting Intestinal Phosphorus Absorption in CKD

Classic phosphorus homeostasis would predict a decrease in intestinal fractional phosphorus absorption in CKD, namely in response to elevated FGF23 and subsequent decrease in 1,25D; i.e. an intestinal compensation for renal decline. In patients with very severe CKD, it appears that intestinal fractional phosphorus absorption is indeed reduced compared with healthy controls (83). However, studies using CKD animal models suggest an absent or insignificant intestinal adaptation to reduce phosphorus burden in CKD. Marks et al. (46) showed that 5/6 nephrectomized (CKD) rats had significantly lower 1,25D, higher PTH, and higher serum phosphorus levels that sham-operated rats (46), a biochemical scenario consistent with late stage CKD in humans. However, fractional phosphorus absorption assessed by in situ ligated loops in the jejunum and duodenum was not statistically different between the CKD rats and control rats, and was inconsistent with the hormonal changes observed (46, 84). Additionally, there was no change of NaPiIIb mRNA in CKD rats compared to controls.

Loghman-Adham (85) et al. modeled a moderate stage CKD by inducing renal failure via two-part 5/6 nephrectomy procedure in rats, and studied the animals when they exhibited increased plasma creatinine and BUN without significant increases in plasma phosphorus. Control and CKD rats were fed either a low phosphorus diet (0.07%) or a high phosphorus diet (0.95%) for 5 days). Results showed that the low phosphorus diet increased phosphorus uptake in brush border membrane vesicles by over a two-fold in the CKD and control rats alike. Consistent with the change in phosphorus uptake, kinetic analysis showed the V_{max} for NaPiIIb was increased by almost three-fold in both control and CKD rats when fed a low phosphorus diet. These rat data suggest that the intestine's ability to adapt to dietary changes in phosphorus may be preserved in the context of moderate renal failure and intestinal fractional phosphorus absorption may not be decreased with decline in kidney function, suggesting a break in the homeostatic regulation of phosphorus in CKD. However, much of the preclinical literature is limited to studies using in vitro or ex vivo methods of phosphorus absorption assessment. Further, few human studies of direct measurement of intestinal phosphorus absorption exist, particularly in CKD. However, Spiegel et al. (64) used 24-hour urine phosphorus excretion as a proxy for phosphorus absorption in the context of controlled feeding have shown that patients with moderate CKD have similar 24-hour urine phosphorus excretion compared with healthy controls (64). Studies of utilizing direct measures of phosphorus absorption are needed to determine how phosphorus absorption is affected by CKD, and, if so, develop translatable strategies to decrease phosphorus absorption in early chronic kidney disease.

References

1. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39:S1-266.
2. Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. Kidney Int. 2011;79(12):1370-8. doi:10.1038/ki.2011.47.
3. Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney international. 2006;69(11):1945-53. doi:10.1038/sj.ki.5000414.

4. Bernasconi R, Aeschbacher S, Blum S, Mongiat M, Girod M, Todd J et al. Fibroblast growth factor 23 and renal function among young and healthy individuals. Clinical chemistry and laboratory medicine. 2018. doi:10.1515/cclm-2017-1183.
5. Gutierrez O. M. MM, Isakova T, Tauh-Hain JA, Tamez H, Shah A, Smith K, Lee H, Thadhani R, Juppner H, Wolf M. Fibroblast Growth Factor 23 and Mortality among Patients Undergoing Hemodialysis. The New England Journal of Medicine. 2008;359:584-92.
6. Berndt T, Kumar R. Novel mechanisms in the regulation of phosphorus homeostasis. Physiology. 2009;24:17-25. doi:10.1152/physiol.00034.2008.
7. Bergwitz C, Juppner H. Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. Annual review of medicine. 2010;61:91-104. doi:10.1146/annurev.med.051308.111339.
8. Medicine Io. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, D.C.: National Academy Press; 1997.
9. Moorthi RN, Moe SM. CKD-mineral and bone disorder: core curriculum 2011. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2011;58(6):1022-36. doi:10.1053/j.ajkd.2011.08.009.
10. Perwad F, Zhang MY, Tenenhouse HS, Portale AA. Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism in vivo and suppresses 25-hydroxyvitamin D-1alpha-hydroxylase expression in vitro. Am J Physiol Renal Physiol. 2007;293(5):F1577-83. doi:10.1152/ajprenal.00463.2006.
11. Lopez-Hilker SDAR, NS; Martin, KJ; Slatopolsky, E. Phosphorus restriction reverses hyperparathyroidism in uremia independent of changes in calcium and calcitriol. Renal Fluid Electrolyte Physiol. 1990;28:F432-F7.
12. Silver J, Naveh-Many T. Phosphate and the parathyroid. Kidney Int. 2009;75(9):898-905. doi:10.1038/ki.2008.642.
13. Walling M. Intestinal Ca and phosphate transport differential responses to vitamin D3 metabolites. Am J Physiol: Endocrinol Metab Gastrointest Physiol. 1977;2:E488-E94.
14. Rizzoli R FH, Bonjour JP. Role of 1,25-Dihydroxyvitamin D3 on Intestinal Phosphate Absorption in Rats with a Normal Vitamin D Supply. Journal of Clinical Investigaion. 1977;60:639-47.

15. Williams KB DH. Characterization of intestinal phosphate absorption using a novel in vivo method. AmJ Physiol Endocrinol Metab. 2007;292:E1917-E21. doi:10.1152/ajpendo.00654.2006.-A.
16. Burnett-Bowie SM, Henao MP, Dere ME, Lee H, Leder BZ. Effects of hPTH(1-34) infusion on circulating serum phosphorushosphate, 1,25-dihydroxyvitamin D, and FGF23 levels in healthy men. J Bone Miner Res. 2009;24(10):1681-5. doi:10.1359/jbmr.090406.
17. Carpenter TO, Imel EA, Holm IA, Jan de Beur SM, Insogna KL. A clinician's guide to X-linked hypophosphatemia. J Bone Miner Res. 2011;26(7):1381-8. doi:10.1002/jbmr.340.
18. Ritter CS, Slatopolsky E. Phosphate Toxicity in CKD: The Killer among Us. Clin J Am Soc Nephrol. 2016;11(6):1088-100. doi:10.2215/CJN.11901115.
19. Cheng CY, Kuro-o M, Razzaque MS. Molecular regulation of phosphate metabolism by fibroblast growth factor-23-klotho system. Advances in chronic kidney disease. 2011;18(2):91-7. doi:10.1053/j.ackd.2010.11.007.
20. Inoue Y, Segawa H, Kaneko I, Yamanaka S, Kusano K, Kawakami E et al. Role of the vitamin D receptor in FGF23 action on phosphate metabolism. Biochem J. 2005;390(Pt 1):325-31. doi:10.1042/BJ20041799.
21. Hasegawa H, Nagano N, Urakawa I, Yamazaki Y, Iijima K, Fujita T et al. Direct evidence for a causative role of FGF23 in the abnormal renal phosphate handling and vitamin D metabolism in rats with early-stage chronic kidney disease. Kidney Int. 2010;78(10):975-80. doi:10.1038/ki.2010.313.
22. Wolf M. Mineral (Mal)Adaptation to Kidney Disease--Young Investigator Award Address: American Society of Nephrology Kidney Week 2014. Clinical journal of the American Society of Nephrology : CJASN. 2015;10(10):1875-85. doi:10.2215/CJN.04430415.
23. Hu MC, Shi M, Cho HJ, Adams-Huet B, Paek J, Hill K et al. Klotho and phosphate are modulators of pathologic uremic cardiac remodeling. J Am Soc Nephrol. 2015;26(6):1290-302. doi:10.1681/ASN.2014050465.
24. Yu J, Deng M, Zhao J, Huang L. Decreased expression of klotho gene in uremic atherosclerosis in apolipoprotein E-deficient mice. Biochemical and biophysical research communications. 2010;391(1):261-6. doi:10.1016/j.bbrc.2009.11.046.

25. Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T et al. Severely reduced production of klotho in human chronic renal failure kidney. Biochemical and biophysical research communications. 2001;280(4):1015-20. doi:10.1006/bbrc.2000.4226.
26. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T et al. FGF23 induces left ventricular hypertrophy. J Clin Invest. 2011;121(11):4393-408. doi:10.1172/JCI46122.
27. Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB, Sr., Gaziano JM et al. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. Archives of internal medicine. 2007;167(9):879-85. doi:10.1001/archinte.167.9.879.
28. Ullrich KMH. Sulfate and phosphate transport in rat kidney proximal tubule. Phil Trans R Soc Lond. 1982;B 299:549-58.
29. Blaine J, Chonchol M, Levi M. Renal control of calcium, phosphate, and magnesium homeostasis. Clin J Am Soc Nephrol. 2015;10(7):1257-72. doi:10.2215/CJN.09750913.
30. Erben RG, Andrukhova O. FGF23 regulation of renal tubular solute transport. Current opinion in nephrology and hypertension. 2015;24(5):450-6. doi:10.1097/MNH.0000000000000145.
31. Biber J, Murer H, Mohebbi N, Wagner CA. Renal handling of phosphate and sulfate. Comprehensive Physiology. 2014;4(2):771-92. doi:10.1002/cphy.c120031.
32. Forster IC, Hernando N, Biber J, Murer H. Phosphate transporters of the SLC20 and SLC34 families. Molecular aspects of medicine. 2013;34(2-3):386-95. doi:10.1016/j.mam.2012.07.007.
33. Hernando N, Wagner CA. Mechanisms and Regulation of Intestinal Phosphate Absorption. Comprehensive Physiology. 2018;8(3):1065-90. doi:10.1002/cphy.c170024.
34. Sabbagh Y, O'Brien SP, Song W, Boulanger JH, Stockmann A, Arbeeny C et al. Intestinal npt2b plays a major role in phosphate absorption and homeostasis. J Am Soc Nephrol. 2009;20(11):2348-58. doi:10.1681/ASN.2009050559.
35. Ghezzi C, Meinild AK, Murer H, Forster IC. Voltage- and substrate-dependent interactions between sites in putative re-entrant domains of a Na(+)-coupled phosphate cotransporter. Pflugers Archiv : European journal of physiology. 2011;461(6):645-63. doi:10.1007/s00424-011-0948-z.

36. Ullrich KJ, Murer H. Sulphate and phosphate transport in the renal proximal tubule. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 1982;299(1097):549-58.
37. Davis GR, Zerwekh JE, Parker TF, Krejs GJ, Pak CY, Fordtran JS. Absorption of phosphate in the jejunum of patients with chronic renal failure before and after correction of vitamin D deficiency. Gastroenterology. 1983;85(4):908-16.
38. Marks J. The role of SLC34A2 in intestinal phosphate absorption and phosphate homeostasis. Pflugers Archiv : European journal of physiology. 2019;471(1):165-73. doi:10.1007/s00424-018-2221-1.
39. Katai K MK, Kishida S, Segawa H, Nii H, Tanaka H, Tani Y, Arai H, Tatsumi S, Morita K, Taketani Y, Takeda E. Regulation of intestinal Na+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dyhydroxyvitamin D3. Biochem J. 1999;343:705-12.
40. Xu H, Bai L, Collins JF, Ghishan FK. Age-dependent regulation of rat intestinal type IIb sodium-phosphate cotransporter by 1,25-(OH)(2) vitamin D(3). Am J Physiol Cell Physiol. 2002;282(3):C487-93. doi:10.1152/ajpcell.00412.2001.
41. Nemere I, Garcia-Garbi N, Hammerling GJ, Winger Q. Intestinal cell phosphate uptake and the targeted knockout of the 1,25D3-MARRS receptor/PDIA3/ERp57. Endocrinology. 2012;153(4):1609-15. doi:10.1210/en.2011-1850.
42. Capuano P RT, Wagner CA, Bacic D, Kato S, Uchiyama Y, St.-Arnoud R, Murer H, Biber J. Intestinal and renal adaptation to a low-Pi diet of type II NaPi cotransporters in vitamin D receptor- and 1alphaOHas-deficient mice. Am J Physiol Cell Physiol. 2004;288:C429-C34. doi:10.1152/ajpcell.00331.2004.-Intake.
43. Lee DBN WM, Brautbar N. Intestinal phosphate absorption: influence of vitamin D and non vitamin D factors. Am J Physiol Gastrointest Liver Physiol. 1986;205:G369-G73.
44. Giral H CY, Sutherland E, Wilson P, Breusegem S, Barry N, Blaine J, Jiang T, Wang X, Levi M. Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphate. Am J Physiol Renal Physiol. 2009;297:F1466-F75. doi:10.1152/ajprenal.00279.2009.-Hyperphosphatemia.
45. Saddoris KL, Fleet JC, Radcliffe JS. Sodium-dependent phosphate uptake in the jejunum is post-transcriptionally regulated in pigs fed a low-phosphorus diet and is independent of dietary calcium concentration. J Nutr. 2010;140(4):731-6. doi:10.3945/jn.109.110080.

46. Marks J, Churchill LJ, Srai SK, Biber J, Murer H, Jaeger P et al. Intestinal phosphate absorption in a model of chronic renal failure. Kidney international. 2007;72(2):166-73. doi:10.1038/sj.ki.5002292.
47. Isakova T, Gutierrez OM, Smith K, Epstein M, Keating LK, Juppner H et al. Pilot study of dietary phosphorus restriction and phosphorus binders to target fibroblast growth factor 23 in patients with chronic kidney disease. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011;26(2):584-91. doi:10.1093/ndt/gfq419.
48. Segawa H KI, Yamanaka S, Ito M, Kuwahata M, Inoue Y, Kato S, Miyamoto K. Intestinal NaPi cotransporter adaptation to dietary phosphorus content in vitamin D receptor null mice. Am J Physiol Renal Physiol. 2003(287):F39-F47.
49. National Kidney Foundation. NKF KDOQI Guidelines; KDOQI Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease. 2003. http://www2.kidney.org/professionals/KDOQI/guidelines_bone/.
50. Uribarri J. Phosphorus homeostasis in normal health and in chronic kidney disease patients with special emphasis on dietary phosphorus intake. Seminars in dialysis. 2007;20(4):295-301. doi:10.1111/j.1525-139X.2007.00309.x.
51. Karp H, Ekholm P, Kemi V, Itkonen S, Hirvonen T, Narkki S et al. Differences among total and in vitro digestible phosphorus content of plant foods and beverages. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2012;22(4):416-22. doi:10.1053/j.jrn.2011.04.004.
52. Waheed AA, Pedraza F, Lenz O, Isakova T. Phosphate control in end-stage renal disease: barriers and opportunities. Nephrol Dial Transplant. 2013;28(12):2961-8. doi:10.1093/ndt/gft244.
53. Murtaugh MA, Filipowicz R, Baird BC, Wei G, Greene T, Beddhu S. Dietary phosphorus intake and mortality in moderate chronic kidney disease: NHANES III. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012;27(3):990-6. doi:10.1093/ndt/gfr367.
54. Hill Gallant KM, Weaver CM, Towler DA, Thuppal SV, Bailey RL. Nutrition in Cardioskeletal Health. Advances in nutrition. 2016;7(3):544-55. doi:10.3945/an.115.011189.

55. Calvo MS, Moshfegh AJ, Tucker KL. Assessing the health impact of phosphorus in the food supply: issues and considerations. Advances in nutrition. 2014;5(1):104-13. doi:10.3945/an.113.004861.
56. Sullivan C, Sayre SS, Leon JB, Machekano R, Love TE, Porter D et al. Effect of food additives on hyperphosphatemia among patients with end-stage renal disease: a randomized controlled trial. JAMA. 2009;301(6):629-35. doi:10.1001/jama.2009.96.
57. de Fornasari ML, Dos Santos Sens YA. Replacing Phosphorus-Containing Food Additives With Foods Without Additives Reduces Phosphatemia in End-Stage Renal Disease Patients: A Randomized Clinical Trial. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2017;27(2):97-105. doi:10.1053/j.jrn.2016.08.009.
58. Mohammad J, Scanni R, Bestmann L, Hulter HN, Krapf R. A Controlled Increase in Dietary phosphate Elevates BP in Healthy Human Subjects. J Am Soc Nephrol. 2018;29(8):2089-98. doi:10.1681/ASN.2017121254.
59. Moorthi RN, Armstrong CL, Janda K, Ponsler-Sipes K, Asplin JR, Moe SM. The effect of a diet containing 70% protein from plants on mineral metabolism and musculoskeletal health in chronic kidney disease. American journal of nephrology. 2014;40(6):582-91. doi:10.1159/000371498.
60. St-Jules DE, Woolf K, Pompeii ML, Kalantar-Zadeh K, Sevick MA. Reexamining the Phosphorus-Protein Dilemma: Does Phosphorus Restriction Compromise Protein Status? Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2016;26(3):136-40. doi:10.1053/j.jrn.2015.12.004.
61. Moe SM, Chertow GM. The case against calcium-based phosphate binders. Clin J Am Soc Nephrol. 2006;1(4):697-703. doi:10.2215/CJN.00560206.
62. Friedman EA. Calcium-based phosphate binders are appropriate in chronic renal failure. Clin J Am Soc Nephrol. 2006;1(4):704-9. doi:10.2215/CJN.01831105.
63. Hill KM, Martin BR, Wastney ME, McCabe GP, Moe SM, Weaver CM et al. Oral calcium carbonate affects calcium but not phosphorus balance in stage 3-4 chronic kidney disease. Kidney international. 2013;83(5):959-66. doi:10.1038/ki.2012.403.

64. Spiegel DM, Brady K. Calcium balance in normal individuals and in patients with chronic kidney disease on low- and high-calcium diets. Kidney international. 2012;81(11):1116-22. doi:10.1038/ki.2011.490.
65. Block GA, Wheeler DC, Persky MS, Kestenbaum B, Ketteler M, Spiegel DM et al. Effects of phosphate binders in moderate CKD. Journal of the American Society of Nephrology : JASN. 2012;23(8):1407-15. doi:10.1681/ASN.2012030223.
66. SM M. Current diagnosis & treatment. Nephrology & hypertension. In: Lerma EV RM, Perazella MA editor. Current diagnosis & treatment. Nephrology & hypertension
New York: McGraw-Hill Medical; 2018. p. 237-54.
67. Gutekunst L. An Update on Phosphate Binders: A Dietitian's Perspective. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2016;26(4):209-18. doi:10.1053/j.jrn.2016.01.009.
68. Beto JA, Ramirez WE, Bansal VK. Medical nutrition therapy in adults with chronic kidney disease: integrating evidence and consensus into practice for the generalist registered dietitian nutritionist. J Acad Nutr Diet. 2014;114(7):1077-87. doi:10.1016/j.jand.2013.12.009.
69. Kidney Disease: Improving Global Outcomes CKDMBDUWG. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney Int Suppl (2011). 2017;7(1):1-59. doi:10.1016/j.kisu.2017.04.001.
70. Brenner BM. Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. Kidney international. 1983;23(4):647-55. doi:10.1038/ki.1983.72.
71. Klahr S, Buerkert J, Purkerson ML. Role of dietary factors in the progression of chronic renal disease. Kidney Int. 1983;24(5):579-87. doi:10.1038/ki.1983.197.
72. Kitada M, Ogura Y, Suzuki T, Sen S, Lee SM, Kanasaki K et al. A very-low-protein diet ameliorates advanced diabetic nephropathy through autophagy induction by suppression of the mTORC1 pathway in Wistar fatty rats, an animal model of type 2 diabetes and obesity. Diabetologia. 2016;59(6):1307-17. doi:10.1007/s00125-016-3925-4.
73. Kitada M, Ogura Y, Monno I, Koya D. A Low-Protein Diet for Diabetic Kidney Disease: Its Effect and Molecular Mechanism, an Approach from Animal Studies. Nutrients. 2018;10(5). doi:10.3390/nu10050544.

74. Scialla JJ, Anderson CA. Dietary acid load: a novel nutritional target in chronic kidney disease? Advances in chronic kidney disease. 2013;20(2):141-9. doi:10.1053/j.ackd.2012.11.001.
75. Metzger M, Yuan WL, Haymann JP, Flamant M, Houillier P, Thervet E et al. Association of a Low-Protein Diet With Slower Progression of CKD. Kidney Int Rep. 2018;3(1):105-14. doi:10.1016/j.ekir.2017.08.010.
76. Klahr S, Levey AS, Beck GJ, Caggiula AW, Hunsicker L, Kusek JW et al. The effects of dietary protein restriction and blood-pressure control on the progression of chronic renal disease. Modification of Diet in Renal Disease Study Group. N Engl J Med. 1994;330(13):877-84. doi:10.1056/NEJM199403313301301.
77. Levey AA, S; Caggiula, AW; England, BK; Greene, T; Hunsicker, LG; Kusek, JW; Rogers, NL; Teschan, PE. Effects of Dietary protein Restriction on the Progression of Moderate Renal Disease in the Modification of Diet in Renal Disease Study. J Am Soc Nephrol. 1996;7:2616-26.
78. Newsome BI, JH; Righiouart, H; Samak, MF; Levey, AS; Beck, GJ; Block, G. Effect of Protein Restriction on Serum and Urine phosphate in the Modification of Diet in Renal Disease (MDRD) Study. American Journal of Kidney Diseases. 2013;61(6):1045-6. doi:10.1053/j.ajkd.2012.12.026
79. National Kidney Foundation. K/DOQI clinical practice guidelines for nutrition in chronic renal failure. Am J Kidney Dis 2000.
80. Chang WX, Xu N, Kumagai T, Shiraishi T, Kikuyama T, Omizo H et al. The Impact of Normal Range of Serum phosphorus on the Incidence of End-Stage Renal Disease by A Propensity Score Analysis. PLoS One. 2016;11(4):e0154469. doi:10.1371/journal.pone.0154469.
81. National Kidney Foundation. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney International Supplements 2017.
82. National Kidney Foundation. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney International Supplements 2012.

83. Farrington K MM, Newman SP, Varghese Z, Moorhead JF. Comparison of radioisotope methods for the measurement of phosphate absorption in normal subjects and in patients with chronic renal failure. Clinical Science. 1979;60(1):55-63.
84. Vorland CJ, Biruete A, Lachcik PJ, Srinivasan S, Chen NX, Moe SM et al. Kidney Disease Progression Does Not Decrease Intestinal Phosphorus Absorption in a Rat Model of Chronic Kidney Disease-Mineral Bone Disorder. J Bone Miner Res. 2019. doi:10.1002/jbmr.3894.
85. M L-A. Renal and Intestinal Pi Transport Adaptation to Low Phosphorus Diet in Uremic Rats. J Am Soc Nephrol. 1993;3:1930-7.

CHAPTER 2: TWENTY-FOUR-HOUR URINE PHOSPHORUS AS A BIOMARKER OF DIETARY PHOSPHORUS INTAKE AND ABSORPTION IN CKD

Published: Stremke ER, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, and Hill Gallant KM. Twenty-Four-Hour Urine Phosphorus as a Biomarker of Dietary Phosphorus Intake and Absorption in CKD. A Secondary Analysis from a Controlled Diet Balance Study. *CJASN*, 2018. 13(7) 1002-1012; DOI: https://doi.org/10.2215.CJN.00390118

Abstract

Background and objectives: Twenty-four-hour urine phosphorus is commonly used as a surrogate measure for phosphorus intake and absorption in research studies, but its reliability and accuracy are unproven in health or chronic kidney disease. This secondary analysis sought to determine the reliability and accuracy of 24-hour urine phosphorus as a biomarker of phosphorus intake and absorption in moderate chronic kidney disease.

Design, setting, participants and measurements: Eight patients with stage 3-4 chronic kidney disease participated in two-week balance studies with tightly-controlled phosphorus and calcium intakes. Thirteen 24-hour urine collections per patient were analyzed for variability and reliability of 24-hour urine phosphorus and phosphorus-to-creatinine ratio. The accuracy of 24-hour urine phosphorus to predict phosphorus intake was determined using a published equation. The relationships of 24-hour urine phosphorus with phosphorus intake, net absorption, and retention were determined.

Results: There was wide day-to-day variation in 24-hour urine phosphorus within and among subjects (CV=30% and 37%, respectively). Two 24-hour urine measures were needed to achieve ≥75% reliability. Estimating dietary phosphorus intake from a single 24-hour urine resulted in underestimation up to 98% in some patients and overestimation up to 79% in others. 24-hour urine phosphorus negatively correlated with whole-body retention, but was not related to net absorption.

Conclusions: From a sample of eight patients with moderate chronic kidney disease on a tightly controlled dietary intake, 24-hour urine phosphorus was highly variable and did not relate to dietary phosphorus intake or absorption, rather it inversely related to phosphorus retention.

Introduction

Dietary phosphorus intake in the United States is high due to both the natural abundance of phosphorus in foods and the use phosphorus-containing food additives (1, 2). Average intakes of phosphorus in healthy adults (3, 4) and adults with chronic kidney disease (CKD) (5) well-exceed 700 mg/d, the recommended dietary allowance for adults ≥19 years-old (6). Phosphorus excess is central to the development and progression of CKD-mineral bone disorder, which is associated with higher fracture, cardiovascular, and mortality risk (7, 8). Many existing and emerging therapies aim to reduce phosphorus absorption via phosphorus intake restriction, binding luminal phosphorus, or direct inhibition of intestinal phosphate transporters. Accurate and reliable assessment of phosphorus intake is needed, but this is complicated by limitations in the tools available (9). These limitations include the nutrient databases that are incomplete and often inaccurate for phosphorus. Differences between phosphorus content determined by nutrient database versus direct chemical analysis of foods show that databases can drastically underestimate phosphorus content of foods (~15% to 70%)(10-16).

Twenty-four-hour urine phosphorus (24-hour urine phosphorus) is considered a good surrogate of phosphorus intake in healthy adults in phosphorus balance because net phosphorus absorption is efficient and is linearly related to intake over a wide range of intakes (6). This relationship has been assumed to also apply to patients with CKD (17, 18) but has not been tested. In CKD, factors other than intake and absorption may affect 24-hour urine phosphorus excretion, including the rate of net bone and tissue retention and volume status (19). While 24-hour urine phosphorus is indisputably useful as a biomarker of absorption in clinical trials with interventions that have a known mechanism to increase or decrease intestinal absorption (i.e. in a randomized controlled trial (RCT) where the intervention is expected to be the cause for effects observed) (20), its reliability and accuracy as a biomarker of phosphorus intake or absorption in observational (associational) studies are unknown. Thus, the aim of this study was to determine the variability and reliability of 24-hour urine phosphorus and its accuracy as a measure of phosphorus intake and absorption in patients with moderate CKD using data from a previously-conducted calcium and phosphorus balance study where phosphorus intake was tightly controlled and precisely measured (21).

Materials and Methods

Study Design and Participants

Data for this secondary-analysis were obtained from a previously-conducted randomized double-blind placebo-controlled cross-over study of the effects of calcium carbonate on calcium and phosphorus balance in n=8 stage 3-4 CKD patients (NCT01161407). Detailed methods and results for the parent study are described elsewhere (21) and in **Supplemental Methods**. Briefly, the parent study consisted of a two-week run-in where all subjects were given 400 IU/day of cholecalciferol, followed by two 3-week study periods separated by a three-week washout period. Each 3-week study period included a 1-week outpatient equilibration period to the controlled diet and assigned treatment, followed by 2-weeks of balance measures in a controlled inpatient setting. Treatment was calcium carbonate given in capsule form t.i.d. with meals or placebo. This study adhered to the Declaration of Helsinki and was approved by the Purdue University and Indiana University Institutional Review Boards. Informed consent was obtained during the parent study.

24-Hour Urine Phosphorus, Urine phosphorus/Creatinine Ratio, and Dietary Phosphorus Intake

During these balance studies, 24-hour urine and feces were collected to calculate calcium and phosphorus balance, which were previously reported (21). 24-hour urine phosphorus was measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 4300DV, Perkin Elmer, %CV=2.4), 24-hour urine creatinine (24-hour urine Cr) excretion was measured by colorimetric assay using a COBAS MIRA clinical analyzer (Roche Diagnostic, Indianapolis, IN, %CV=2.5), and 24-hour urine phosphorus-to-creatinine ratio (24-hour urine phosphorus/Cr) was calculated. Whole-body phosphorus balance was calculated as daily intake minus fecal and urine excretion averaged over the entire balance period. Net phosphorus absorption was calculated as daily intake minus fecal excretion averaged over the entire balance period. Intake was controlled, with menus designed by a registered dietitian and prepared in a metabolic kitchen. Phosphorus intake from the controlled 4-day cycle menu was 1564±52 mg/d, calcium was 957±23 mg/d and sodium was 2749±541 mg/d (menu details provided in a previously published supplemental file(21)). Mineral content of the controlled diets was analyzed from ashed, homogenized diet composites by ICP-OES. “Bone balance” was determined from full kinetic modeling of oral and intravenous ^{45}Ca tracers and reflects a late turnover calcium pool that is

generally regarded as bone turnover, but that does not distinguish from potential extraskeletal calcification. Fasting serum and urine biochemistries were obtained at four time-points during the study: baseline, end of the diet equilibration week, and the end of each week of balance (i.e. four measures over 3 weeks, each a week apart). Estimated eGFR (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) study equation (22). Intact fibroblast growth factor 23 (iFGF23) was measured by enzyme-linked immunosorbent assay (Kainos Laboratories, Tokyo, Japan) and intact parathyroid hormone (iPTH) by immunoradiometric assay (N-tact, Diasorin, Stillwater, MN). Serum phosphorus and creatinine were measured by colorimetric assay using a COBAS MIRA clinical analyzer (Roche Diagnostic). A poorly-absorbable fecal marker (polyethylene glycol, PEG m.w.3500) was given 3/g/day to assess fecal collection adherence and to calculate daily fecal calcium and phosphorus-to-PEG ratios (Ca:PEG and P:PEG), reflective of daily net absorption, and used to indicate achievement of steady-state for balance measures (23-25).

Statistical Methods

All statistical analyses were performed using Statistical Analysis Software (SAS) v9.3 (Cary, NC), and statistical significance was set $\alpha=0.05$. Descriptive statistics were performed for variability in 24-hour urine phosphorus, phosphorus intake, and 24-hour urine phosphorus/Cr. Within subject variation was determined from the 13 consecutive days of data during the balance periods. Among subject variation was determined using average values for each subject over the 13-days of data. These data were complete for all subjects. Standard deviations and %CVs were calculated and are reported to describe the variation (**Supplemental Methods**). For 24-hour urine phosphorus and 24-hour urine phosphorus/Cr, reliability, the correlation between two replicates on the same individual (i.e. the consistency of a measure), was estimated by the intraclass correlation coefficient. The Spearman-Brown prediction formula (26) was used to calculate reliability for the average of different numbers of replicates and to determine the number of replicates needed to achieve a reliability of at least 75% (**Supplemental Methods**). Linear regression was used to determine correlations with 95% confidence intervals for whole-body phosphorus retention, “bone balance” (from calcium kinetics), and net phosphorus absorption with 24-hour urine phosphorus.

To assess the accuracy of 24-hour urine phosphorus as an indicator of dietary phosphorus intake, we calculated an estimated phosphorus intake from each daily 24-hour urine phosphorus using the published equation of Lemann (27) ($P_{urine(mmol/d)} = 1.73 + 0.512* P_{intake(mmol/d)}$), as referenced in the Institute of Medicine Dietary Reference Intakes report on phosphorus (6). These calculated values were compared with the actual phosphorus intake values measured in the ashed, homogenized diet composites from the balance study, and percent over- or underestimation are reported. Variation around the mean of calculated intake was expressed in standard deviations and %CV.

We performed similar analyses for 24-hour urine calcium (24-hour urine Ca) and calcium-to-creatinine ratio (24-hour urine Ca/Cr), fecal phosphorus and calicum, and fecal P:PEG and Ca:PEG ratios to describe the variability and reliability of these measures within and among subjects. Analyses were performed on data from both the placebo and calcium carbonate, but results were similar and calcium carbonate did not affect the level of variation observed in 24-hour urine phosphorus. Results from analyses on data from the placebo balance period on all patients are reported here.

Results

Baseline Characteristics and Steady-State

Baseline characteristics are given in **Table 2.1.** After the one-week equilibration period to the controlled diet, both fecal Ca:PEG and fecal P:PEG became consistent (i.e. showed no directional trend) indicating the patients had achieved steady-state for calcium and phosphorus balance measures (**Supplemental Figure S2.1A,B**) (23, 24).

Variation in 24-Hour Urine Phosphorus, Urine Creatinine, and Urine Phosphorus/Creatinine Ratio

Wide within and among subject variation in 24-hour urine phosphorus was observed. Standard deviations within subjects averaged 186 mg/d and ranged from 105-248 mg/d (%CV=30, range 15-60%) and among subjects of 268 mg/d (%CV=37) (**Table 2.2, Figure 2.1A,B**). Despite close monitoring by clinical research center staff, average standard deviation within subjects for urine creatinine was 174 mg/d (range 94-337 mg/d;%CV = 15, range 9-28%) (**Table 2.2, Figure**

2.1C,D). Among subject standard deviation for urine creatinine was 427 mg/d (%CV=34), reflecting the range in body size and eGFR. The variation in 24-hour urine phosphorus/Cr ratio (%CV=27, range 12-51%) was similar compared with 24-hour urine phosphorus (**Table 2.2, Figure 2.1E,F**). Within subject variation in eGFR taken from four time points throughout the study was mean CV=10% (range 2-37%), and among subjects was CV=16% (**Table 2.2**).

Reliability of 24-Hour Urine Phosphorus and Phosphorus-Creatinine Measures

The variation in 24-hour urine phosphorus and P/Cr was used to determine the number of 24-hour replicates needed to obtain a reliable measure. The intraclass correlation coefficient for 24-hour urine phosphorus was rho=0.65 (95%CI: 0.42,0.89), and for P/Cr, rho=0.60 (95%CI: 0.36,0.87). From these values, the number of replicates needed to ≥75% reliability for 24-hour urine phosphorus or P/Cr was 2 (95% CI: 1,5), and more replicates for a greater degree of reliability (**Figure 2.2A,B**).

Accuracy of 24-Hour Urine Phosphorus in Predicting Dietary Phosphorus Intake

A published equation (6, 27) was used to determine the accuracy of 24-hour urine phosphorus to predict dietary phosphorus intake. Predicted intakes from this equation both underestimated and overestimated the known P intake by as much as 98% and 79%, respectively (explained solely by the variation in 24-hour urine phosphorus, as the only variable entered into this equation). The lowest predicted P intake was <40 mg/d, and the highest was >2800 mg/d (**Figure 2.1G**).

Correlations with 24-Hour Urine Phosphorus and Other Related Measures

The relationships between mean 24-hour urine phosphorus for each subject and net phosphorus absorption, fecal phosphorus, whole-body phosphorus retention (i.e. phosphorus balance), "bone balance" (from calcium kinetics), serum iPTH, and serum iFGF23 were determined. 24-hour urine phosphorus was highly negatively correlated with whole-body P retention (r= -0.88, p=0.004, **Table 2.2, Figure 2.3A**) and with "bone balance" from calcium kinetics (r= -0.84, p=0.009). There were no statistically significant correlations with net phosphorus absorption (r= -0.15, p=0.73, **Table 2.2, Figure 2.3B)**, fecal phosphorus (r=0.28,

p=0.51), serum iPTH (r=0.55, p=0.16), nor serum iFGF23 (r=0.53, p=0.18), although small sample size may be limiting power to detect these relationships.

The wide day-to-day variation within individuals was also investigated further. 24-hour urine phosphorus was again negatively associated with whole-body phosphorus retention (partial r= -0.29, p=0.001), and not related to daily net phosphorus absorption or fecal P:PEG. However, greater urine volume was related to greater 24-hour urine phosphorus (partial r=0.46, p<0.001).

Variation and Reliability of 24-Hour Urine Calcium and 24-Hour Urine Calcium/Creatinine Ratio Measures

24-hour urine calcium, which was very low in these patients and did not increase even when given an additional 1500 mg/d by binders, as previously reported (21), did not relate to measures of whole-body calcium retention (r= -0.28, p=0.51), net calcium absorption (r= -0.02, p=0.96), fecal calcium excretion (r= -0.06, p=0.89), nor bone balance (r= -0.47, p=0.24). Wide within and among subject variation in 24-hour urine calcium was observed, but this must be interpreted in the context of very low mean 24-hour urine calcium output in most of these patients (in contrast to 24-hour urine phosphorus) (**Figure 2.4A-D**). Standard deviations within subjects averaged 13 mg/d and ranged from 2 to 28 mg/d (%CV=58, range 19-107%) and among subjects was 53 mg/d (%CV=116). The variation in 24-hour urine Ca/Cr within subjects averaged 0.01 and ranged from 0.001 to 0.02 (%CV=57, range 18-120%) and among subjects was 0.04 (%CV=85%). The intraclass correlation coefficient for 24-hour urine calcium was rho=0.92 (95%CI: 0.82,0.98), and for Ca/Cr, rho=0.92 (95%CI: 0.83,0.98). Thus, the number of replicates needed for ≥75% reliability for 24-hour urine calcium or urine Ca/Cr was only 1.

Discussion

The results of this study show that 24-hour urine phosphorus is a highly variable measurement, even under optimal clinical research center conditions for complete and timed collections, and that repeated measurements are necessary when a reliable value is needed. The variability in a less-controlled outpatient setting would almost certainly be higher. 24-hour urine Cr was also variable day-to-day within subjects in this controlled setting. This could potentially indicate a lack of steady-state in creatinine metabolism. But, if this were the case, one would expect to see a trend in values. Instead, we saw random fluctuation, indicating this was random variation,

produced by biological and/or methodological factors. There have been reports of tighter variation in day-to-day 24-hour urine Cr excretion in healthy adults (28), but there have also been reports of similar variation in healthy adults (29-31) compared with the moderate CKD patients in the present study. Healthy adolescents have shown even greater day-to-day variation in a controlled research setting (32). A study of patients with diabetic nephropathy showed similar random day-to-day variation in 24-hour urine Cr compared with our study as well (%CV = 27.7%) (33). Some of the variation in within-individual 24-hour urine phosphorus and urine Cr may be due in part to variation in complete urinary tract and bladder emptying. However, correcting for urine creatinine did not lessen the within subject variation in 24-hour urine phosphorus, as shown by the similar extent of variation in 24-hour urine phosphorus/Cr as 24-hour urine phosphorus. Another possibility to explain this variation is day-to-day fluctuations of glomerular filtration rate (GFR). Data on daily GFR in these subjects was not available over the 13 days. However, eGFR was determined in each patient using morning fasting serum creatinine values at four time-points during the study. The mean %CV in eGFR observed in our study was similar in extent to the mean CV of 13.8% for GFR (measured directly by iothalamate clearance) reported in a subset of participants in the Chronic Renal Insufficiency Cohort (34). Caloric consumption was controlled day-to-day for each patient, and patients had high adherence to their study diets, so this is likely not a source of variation in either 24-hour urine phosphorus or urine creatinine. Body weights were also stable over the study. Daily fluctuations in blood pressure may contribute to variation in urine flow and thus solute excretion (33, 35). Although the total amount of dietary phosphorus was controlled day-to-day, the potential differences in the relative bioavailability of phosphorus from various food sources could have contributed to the variation observed. However, if this were the case, we would expect to have seen a pattern of association between 24-hour urine phosphorus excretion and the cycle menu day, which we did not. Methodological errors are always potential contributors to variation, including incomplete, poorly timed, or inaccurately portioned collections. However, we have no reason to suspect these played large roles in our study due to protocols that were in place to minimize these errors.

We show that the average of at least two 24-hour urine phosphorus measures are required for a reliable measure with at least 75% reliability. While 75% is generally considered a threshold for an acceptable level of reliability (26), the 13 replicates that were available from these balance studies gave >90% reliability for both 24-hour urine phosphorus and P/Cr. However, the wide

variation in the 13-day mean 24-hour urine phosphorus among subjects (**Table 2.2**) indicates that even a reliable measurement of 24-hour urine phosphorus could lead to erroneous inferences regarding phosphorus intake and absorption. To illustrate this point, we show both the large overestimation and underestimation of dietary phosphorus intake possible when calculated based on 24-hour urine phosphorus using a published equation (6, 27). Thus, our results show that for an individual patient, 24-hour urine phosphorus was not reflective of actual phosphorus intake, despite a relatively high correlation (r=0.88) between 24-hour urine phosphorus and intake reported in the literature across pooled studies of the general population (27). Recently, large among-subject variation in 24-hour urine phosphorus was also reported in a study of eight young healthy Japanese men studied on a fixed phosphorus diet (1,138 mg/d) over five days of 24-hour urine collections (36), which corroborates our findings.

Similar variability has been reported for 24-hour urine sodium collections in healthy individuals in a controlled environment, attributed in part due to oscillatory secretion of mineralocorticoid that did not align to a 24-hour day and excretion of other solutes that might affect urinary osmolarity and thus sodium excretion (37). A similar phenomenon may occur with urinary phosphorus excretion which is regulated by GFR, urine volume, sodium reabsorption, and two hormones PTH and FGF23 and their regulator calcitriol. Indeed, the relationship within subjects between higher 24-hour urine phosphorus and greater urine volume suggests that this variation may be due to daily variation in GFR, however we did not have daily GFR data from this study to confirm this hypothesis. There is also diurnal variation in urinary phosphorus excretion (38). Furthermore, after an acute oral phosphorus load, PTH rises after 8 hours, FGF23 rises after 12 hours, and calcitriol decreases at 36 hours. Thus, it is likely that fluxes in these hormones do not adhere to a 24-hour clock resulting in variability of phosphorus excretion (39). We were limited by the number of sampling time points available to assess the relationship among 24-hour urine phosphorus excretion and diurnal and day-to-day variation in FGF23 or PTH.

Our results also suggest that differences in whole-body phosphorus retention, not differences in intestinal phosphorus absorption, were responsible for the wide variation observed among these eight patients consuming the same fixed level of dietary phosphorus. This may be due to differences in bone remodeling, extraskeletal tissue uptake, or kidney efficiency for phosphorus excretion. Indeed, the patient with the *lowest* average 24-hour urine phosphorus (225 mg/d) had a net phosphorus absorption of 903 mg/d (60% of intake), and the *highest* value for

whole-body phosphorus retention (680 mg/d). Conversely, the patient with the *highest* average 24-hour urine phosphorus (1083 mg/d) had a similar level of net phosphorus absorption (845 mg/d; 54% of intake), but *negative* whole-body phosphorus retention (-231mg/d) **(Table 2.2**). Based solely on 24-hour urine phosphorus, one would have concluded that former patient had very low phosphorus intake and/or absorption and the latter had very high phosphorus intake and/or absorption. Neither conclusion would have been correct. Importantly, serum phosphorus did not relate to whole-body phosphorus balance (**Table 2.2**) (21). Within subjects, 24-hour urine phosphorus was also negatively associated with whole-body phosphorus balance and not net phosphorus absorption. These results are corroborated by a recently published study by Turner et al. (40) which showed reduced urinary phosphorus excretion in adenine-induced CKD rats compared to healthy rats that was not associated with a reduction in dietary phosphorus absorption, assessed by oral gavage of P-33 tracer. Turner et al. argue similarly that tissue retention, not impaired absorption, was responsible for the lower urine phosphorus values seen in CKD.

These results provide an alternative explanation for unexpected associations with 24-hour urine phosphorus in CKD patients in the literature. For example, Palomino et al. (17) showed that higher 24-hour urine phosphorus was associated with lower, not higher, risk of cardiovascular events. Similarly, a post-hoc analysis of MDRD study data (41) showed that 24-hour urine phosphorus was not related to averse outcomes in CKD patients, which was counter to the hypothesis based on using 24-hour urine phosphorus as a proxy of phosphorus intake/absorption. If higher 24-hour urine phosphorus is indicative of lower phosphorus retention, rather than higher phosphorus intake/absorption, then inverse relationships are expected rather than surprising.

Importantly, the results presented here underscore an important and underappreciated facet to the results from our primary paper from these balance studies (21): the *range* in absolute phosphorus balance values among these eight patients. The primary study was designed to evaluate the effect of a calcium-based phosphate binder on calcium and phosphorus balance. As such, means and standard errors were reported indicating phosphorus balance was neutral on average and was not affected by the calcium-based phosphate binder. Here we show that these eight patients spanned a wide range from negative to positive phosphorus balance values. Balance data from a larger sample of patients are needed to determine a “true” range in phosphorus balance in this population.

In comparison to 24-hour urine phosphorus, 24-hour urine calcium also showed high %CV values, however this was mainly due to the very low mean 24-hour urine calcium output in most of these patients. Thus, this was not particularly meaningful variation (for instance, when mean 24-hour urine calcium excretion is 8 mg/d with a standard deviation of 8 mg/d and 100% CV). Therefore, it is not surprising that 24-hour urine calcium did not relate to net calcium absorption nor whole-body calcium balance. This presents a challenge in assessing calcium load, absorption, or retention in CKD patients, as neither urinary calcium nor serum calcium are appropriate biomarkers for intake or absorption (21, 42). At present, the most useful and clinically available assessment of calcium load may be a careful evaluation of calcium intake from dietary, supplemental, and pharmacological sources (43).

Fecal phosphorus and calcium excretion was highly variable day-to-day within individuals (**Supplemental Results**). Adjustment for fecal PEG output greatly reduced the variability and thus improved the reliability for fecal calcium and phosphorus expressed as a mg:mg ratio with fecal PEG. Following a 1-week diet equilibration period, 2 and 6 replicates are needed for ≥75% reliability in fecal P:PEG and Ca:PEG, respectively. Fecal PEG adjustments can over-correct for mineral excretion (23), as there is some variation among individuals for intestinal PEG (m.w. 3350) absorption (44). Thus, for longer balance periods (e.g. 2 weeks or longer), unadjusted fecal calcium and phosphorus values averaged over the balance period may be preferable for use in balance equations, and fecal Ca:PEG and P:PEG ratios used only for assessment of steady-state and fecal PEG % recovery as an indicator of adherence (e.g.>80% recovery considered as high adherence). However, for studies with shorter balance periods (e.g. 48-hour studies), PEG adjustment would be highly preferable to the alternative of no adjustment. In these cases, we recommend that authors report unadjusted mg/day of fecal calcium and/or phosphorus, along with a PEG-adjusted value to be used in balance calculations, representing the amount of oral PEG administered per day (e.g. mg fecal P per 3g fecal PEG recovery). It important to emphasize the need for an adequate diet equilibration period of ≥1 week that includes oral PEG administration prior to balance measurements.

Strengths of our study include the well-controlled diet and 2-week balance study in a controlled setting with 13 days of 24-hour urine collections. However, the data come from only eight CKD patients, so generalizability is limited. Our results also cannot be applied to the general population with preserved kidney function. The potential reasons for heterogeneity in 24-hour

urine phosphorus among individuals and day-to-day variation within individuals are numerous and may include race, sex, body-mass-index, caloric intake, diabetes status, and baseline intake of medications such as proton-pump inhibitors or diuretics. Thus, investigation into the contributions of such factors would require larger sample sizes of a heterogeneous population of kidney disease patients, as our small sample does not allow us to parse out the effects of these factors. The lack of a range of controlled phosphorus intakes limits our ability to show relationships with varying intake. Only phosphorus, calcium, and creatinine were measured in the urine samples in the original study, so variability and reliability of additional urine solutes is not available for description or comparison.

These results do not preclude using change in 24-hour urine phosphorus as an outcome related to phosphorus absorption in interventional studies with treatments that have a known mechanism impacting phosphorus absorption (e.g. phosphate binders (45) or low phosphorus diets (46, 47)). This is due to experimental study design where it is appropriate to infer cause and effect resultant of the intervention, particularly when there is a biological mechanism that supports the cause and effect relationship. Instead, our results suggest that caution must be used in interpreting 24-hour urine phosphorus in observational studies or in individual patients in the absence of intervention. Instead of considering 24-hour urine phosphorus as an estimate of phosphorus absorption, our results suggest it should be considered a reflection of whole-body phosphorus retention. But, these conclusions require confirmation from additional studies of more CKD patients including age/sex/race-matched controls with preserved kidney function.

Table 2.1 Patient Demographics and Baseline Characteristics†

Characteristic	Value
Female, n	2/8
Black, n	5/8
Diabetes present, n	6/8
Hypertension present, n	8/8
Age, years	59 ± 7
Body mass index, kg/m^2	38.7 ± 8.7
eGFR, ml/min per 1.73m^2	36 ± 9
Serum calcium, mg/dL	9.6 ± 0.3
Serum phosphorus, mg/dL	3.8 ± 0.6
Serum Parathyroid hormone, pg/mL	85 ± 59
Serum intact FGF23, pg/mL	79 ± 40

†Values are means ± SD unless otherwise noted. eGFR, estimated glomerular filtration rate; FGF23, fibroblast growth factor 23.

Table 2.2 Within and Among Subject Variability in Phosphorus and Creatinine Measures

Within Subjects	24-hour urine P (mg/d) †	24-hour urine Cr (mg/d) †	24-hour urine P/Cr (mg/mg) †	P balance (mg/d) ◊	Net P Abs (mg/d) ◊	%Net P Abs (%) ◊	Serum P (mg/dL) ‡	Serum Cr (mg/dL) ‡	eGFR (mL/min) ‡	Serum iPTH (pg/mL)	Serum iFGF23 (pg/mL)
Subject 1	991 (248) *25%*	1911 (168) *9%*	0.52 (0.12) *23%*	-260	731	47	3.8 (0.3) *8.4%*	3.0 (0.1) *2.3%*	29 (0.6) *2%*	143 (55) *39%*	74 (12) *16%*
Subject 2	682 (105) *15%*	1023 (94) *9%*	0.67 (0.08) *12%*	-229	452	29	3.8 (0.4) *9.6%*	1.9 (0.1) *7.4%*	34 (3) *10%*	47 (10) *27%*	101 (6) *5%*
Subject 3	1083 (209) *19%*	1277 (140) *11%*	0.84 (0.10) *12%*	-231	845	54	4.0 (0.5) *13.1%*	1.5 (0.1) *5.0%*	37 (2) *7%*	60 (10) *15%*	135 (8) *18%*
Subject 4	843 (127) *17%*	1180 (135) *11%*	0.71 (0.10) *13%*	82	925	59	4.0 (0.5) *13.4%*	1.7 (0.2) *10.0 %*	31 (4) *11%*	72 (9) *14%*	62 (28) *50%*
Subject 5	693 (243) *35%*	1555 (182) *12%*	0.44 (0.14) *32%*	181	872	53	3.6 (0.5) *14.5%*	1.7 (0.05) *2.9%*	41 (1) *2%*	59 (4) *10%*	50 (23) *31%*
Subject 6	535 (158) *30%*	771 (215) *28%*	0.69 (0.26) *38%*	344	901	63	3.2 (0.5) *15.2%*	1.7 (0.1) *7.0%*	41 (3) *7%*	51 (11) *22%*	74 (14) *20%*
Subject 7	708 (241) *34%*	1691 (337) *20%*	0.43 (0.14) *32%*	203	923	60	3.7 (1.3) *34.1%*	3.4 (0.9) *25.9%*	26 (9) *37%*	64 (10) *23%*	62 (26) *50%*
Subject 8	225 (136) *60%*	731 (123) *17%*	0.31 (0.15) *51%*	680	903	60	3.8 (0.3) *6.8%*	1.9 (0.1) *3.2%*	30 (1) *5%*	44 (9) *30%*	61 (15) *36%*
Among Subjects:											
Mean (SD)	720 (268)	1268 (427)	0.58 (0.18)	96 (330)	819 (161)	53 (11)	3.7 (0.3)	2.1 (0.7)	34 (5)	67 (32)	77 (28)
CV	*37%*	*34%*	*31%*	*343%*	*20%*	*21%*	*7%*	*33%*	*16%*	*47%*	*36%*

†Values for each subject are mean (SD) and *CV%* of 13 days of values in that individual for 24-hour urine phosphorus, 24-hour urine Cr, 24-hour urine phosphorus/Cr, and predicted P intake from daily 24-hour urine phosphorus values. ‡Values for serum phosphorus, Cr, eGFR, iPTH, and iFGF23 are mean (SD) and *CV%* of four values in that individual taken at baseline, end of the 1 week equilibration period to the diet, and at the end of each week of the balance period (i.e. 4 values over 3 weeks, in 1 week intervals). eGFR was calculated with the MDRD study equation. ◊Single values of Phosphorus balance, Net P Abs and % Net P Abs are presented for each subject that are calculated using data from the entire balance period.

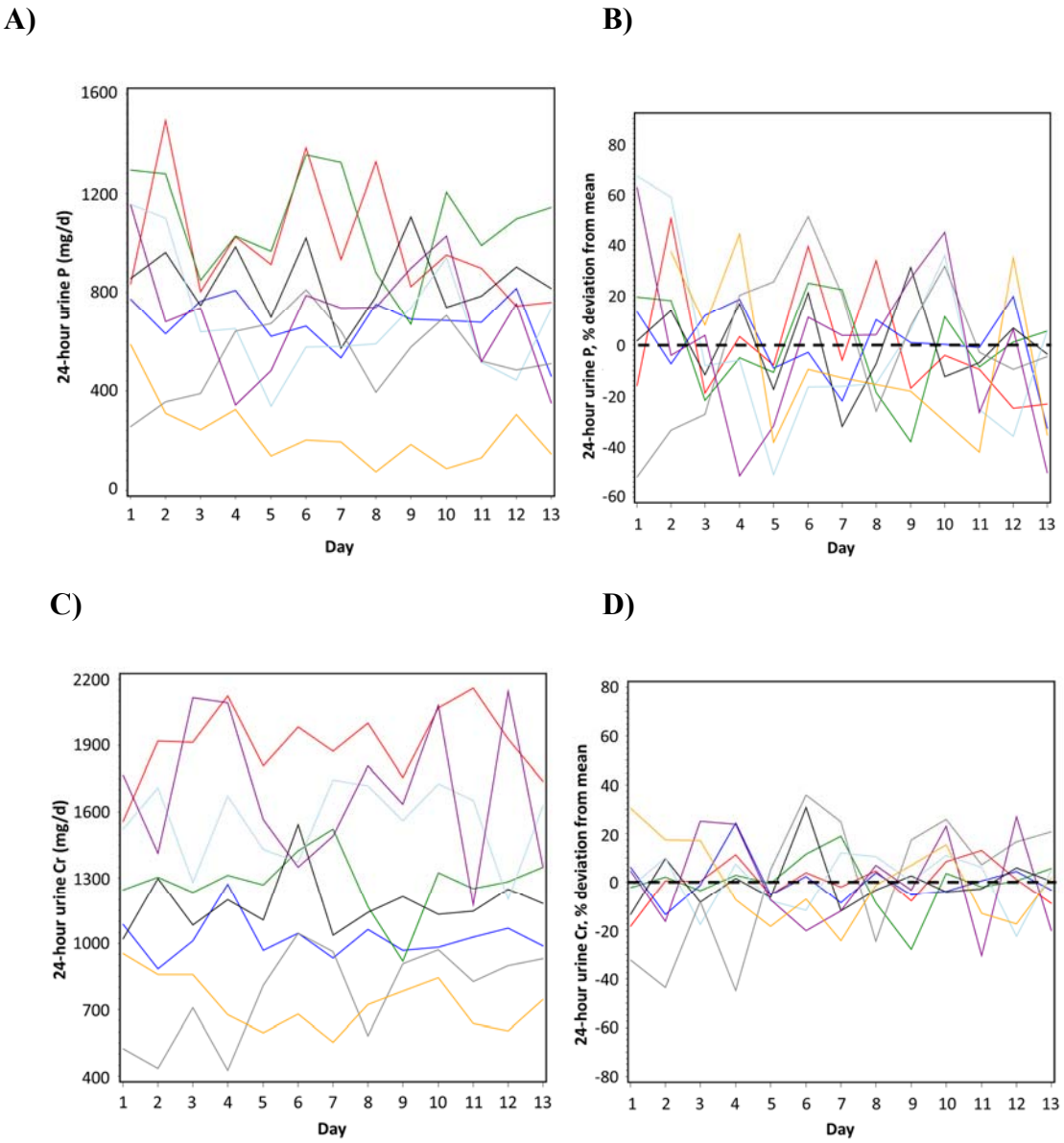
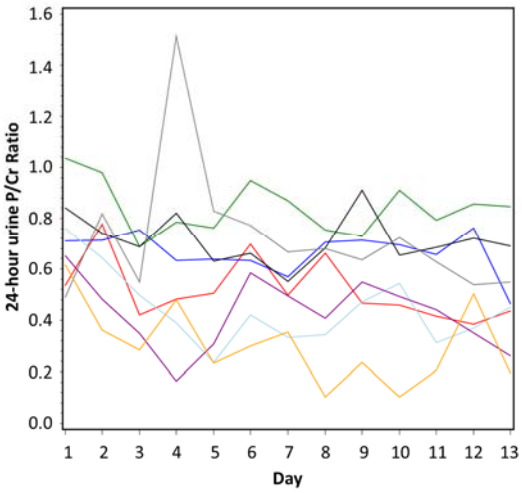


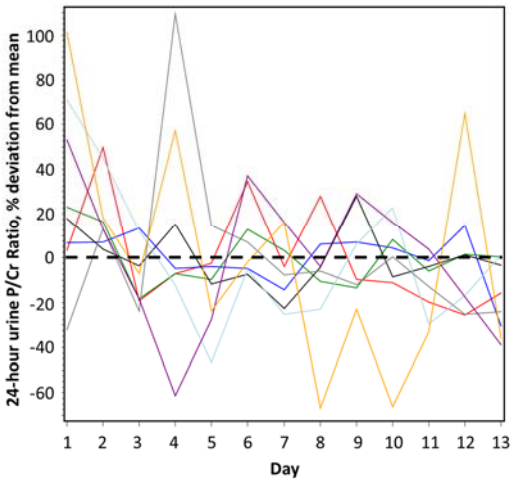
Figure 2.1 Daily variation in subjects urine phosphorus, creatinine, and predicted phosphorus intake. Daily variation in subjects in **A)** 24-hour urine phosphorus (absolute values) and **B)** 24-hour urine phosphorus (% variation above and below the 13-day-mean (set at zero) for each subject); **C)** 24-hour urine Cr (absolute values) and **D)** 24-hour urine Cr (% variation above and below the 13-day-mean (set at zero) for each subject) ; **E)** 24-hour urine phosphorus/Cr ratio (absolute values) and **F)** 24-hour urine phosphorus/Cr (% variation above and below the 13-day-mean (set at zero) for each subject); and **G)** predicted dietary phosphorus intake calculated based on 24-hour urine phosphorus (see methods). In panel G, the measured, controlled level of P intake is shown by the horizontal black line. In panels B, D, and F, the mean for each subject is set at zero and the % fluctuation each day above or below the mean is shown; zero is indicated by a horizontal black dashed line. In all panels, different color lines represent individual subjects.

Figure 2.1 continued

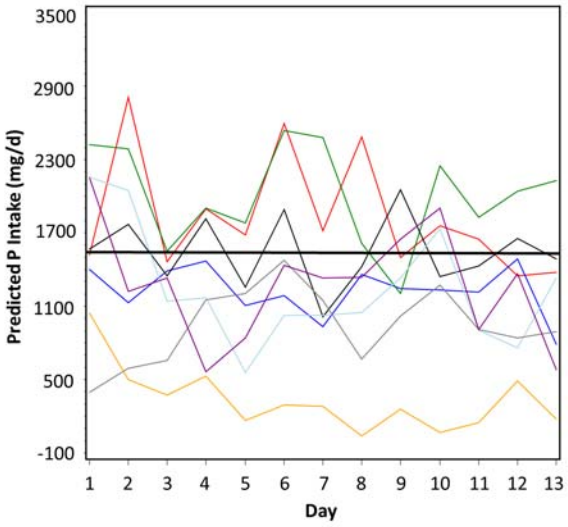
E)



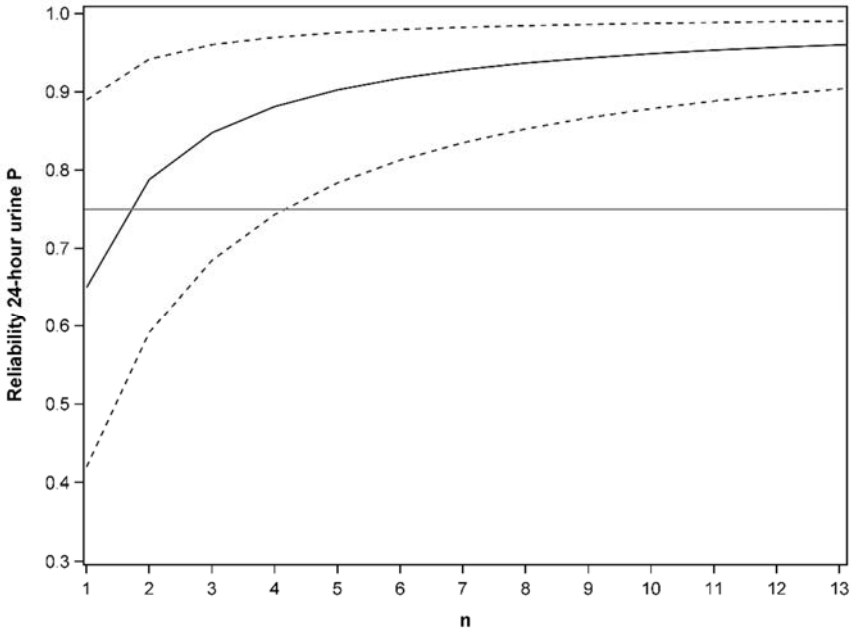
F)



G)



A)



B)

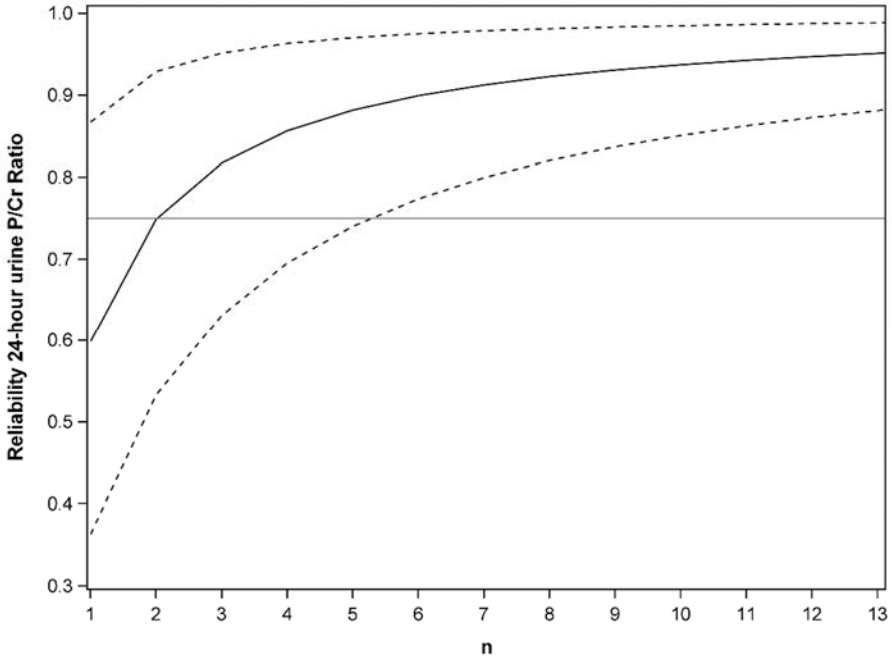
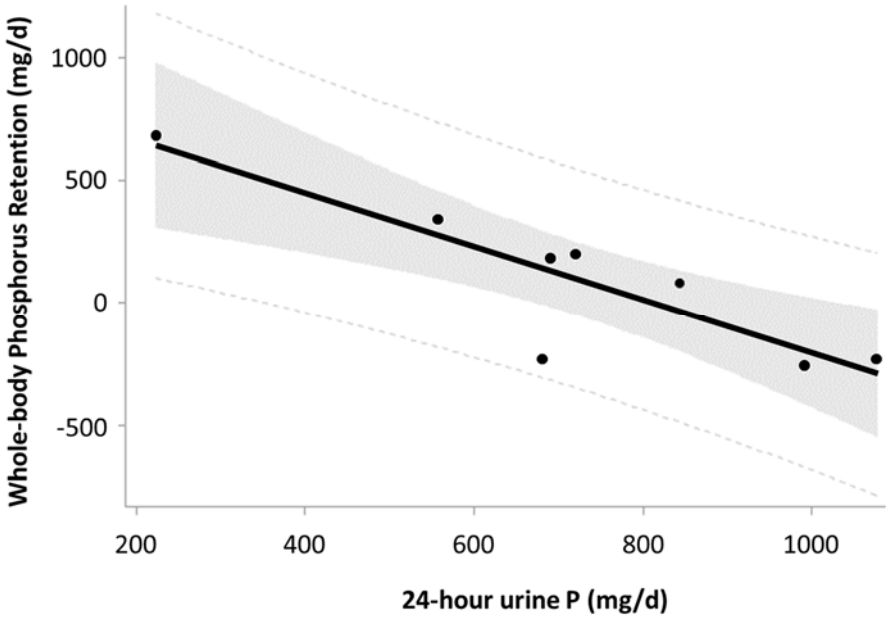


Figure 2.2 Reliability of **A)** 24-hour urine phosphorus and **B)** 24-hour urine phosphorus/Cr. "n" is the number of 24-hour urine collections needed to achieve various levels of reliability. The solid line represents the estimated reliability with n samples, and 95% confidence intervals around this estimate is indicated by dashed lines. Reliability is affected by the variability of the measure, and is the extent to which a measurement is predicted to give the same result when repeated.

A)



B)

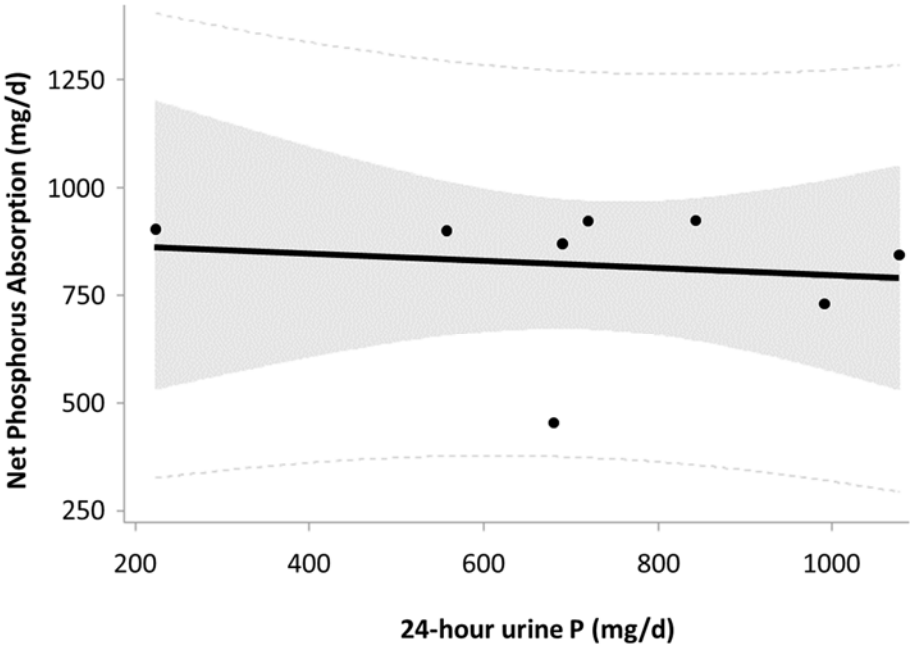


Figure 2.3 Correlations between **A)** 24-hour urine phosphorus and whole-body P retention and **B)** 24-hour urine phosphorus and net P absorption. Solid line is the regression fit; shaded area is the 95% confidence limits; and the dotted lines indicate the 95% prediction limits. The regression equation for panel A is: Retention (mg/d) = 833 - 1.088*(24-hour urine phosphorus, mg/d). Note that this equation could be used to estimate phosphorus retention from 24-hour urine phosphorus (ideally ≥2 replicates) with the caveat that subjects are consuming a similar phosphorus intake of ~1,500 mg/day as those in this study.

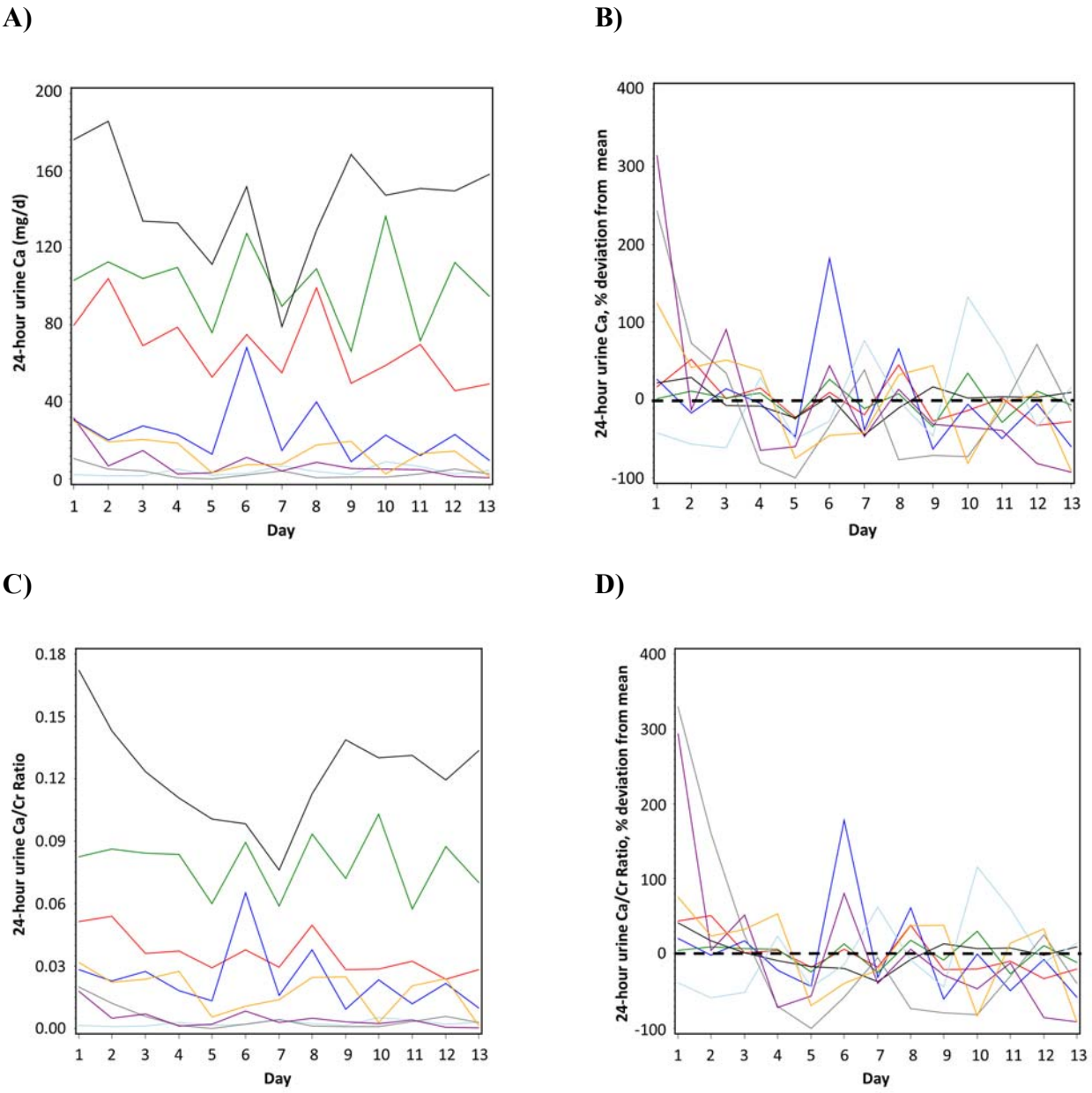


Figure 2.4 Daily variation in subjects in **A)** 24-hour urine Ca (absolute values) and **B)** 24-hour urine Ca (% variation above and below the mean (set at zero) for each subject); **C)** 24-hour urine Ca/Cr ratio (absolute values) and **D)** 24-hour urine Ca/Cr (% variation). In panels B and D, the mean for each subject is set at zero and the % fluctuation each day above or below the mean is shown; zero is indicated by a horizontal black dashed line. In all panels, different color/ lines represent individual subjects.

References

1. Uribarri J: Phosphorus homeostasis in normal health and in chronic kidney disease patients with special emphasis on dietary phosphorus intake. *Semin Dial,* 20**:** 295-301, 2007
2. Karp H, Ekholm P, Kemi V, Itkonen S, Hirvonen T, Narkki S, Lamberg-Allardt C: Differences among total and in vitro digestible phosphorus content of plant foods and beverages. *J Ren Nutr,* 22**:** 416-422, 2012
3. Chang AR, Lazo M, Appel LJ, Gutierrez OM, Grams ME: High dietary phosphorus intake is associated with all-cause mortality: results from NHANES III. *Am J Clin Nutr,* 99**:** 320-327, 2014
4. Calvo MS, Park YK: Changing phosphorus content of the U.S. diet: potential for adverse effects on bone. *J Nutr,* 126**:** 1168S-1180S, 1996
5. Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H, Appleby D, Nessel L, Bellovich K, Chen J, Hamm L, Gadegbeku C, Horwitz E, Townsend RR, Anderson CA, Lash JP, Hsu CY, Leonard MB, Wolf M: Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int,* 79**:** 1370-1378, 2011
6. Institute of Medicine. DRI: Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, DC, National Academy Press, 1997
7. Wolf M: Mineral (Mal)Adaptation to Kidney Disease--Young Investigator Award Address: American Society of Nephrology Kidney Week 2014. *Clin J Am Soc Nephrol,* 10**:** 1875-1885, 2015
8. Pool LR, Wolf M: FGF23 and Nutritional Metabolism. *Annu Rev Nutr,* 37**:** 247-268, 2017
9. Hill Gallant KM: Studying dietary phosphorus intake: the challenge of when a gram is not a gram. *Am J Clin Nutr,* 102**:** 237-238, 2015
10. Benini O, D'Alessandro C, Gianfaldoni D, Cupisti A: Extra-phosphate load from food additives in commonly eaten foods: a real and insidious danger for renal patients. *J Ren Nutr,* 21**:** 303-308, 2011
11. Carrigan A, Klinger A, Choquette SS, Luzuriaga-McPherson A, Bell EK, Darnell B, Gutierrez OM: Contribution of food additives to sodium and phosphorus content of diets rich in processed foods. *J Ren Nutr,* 24**:** 13-19, 19e11, 2014

12. Moreno-Torres R, Ruiz-Lopez MD, Artacho R, Oliva P, Baena F, Baro L, Lopez C: Dietary intake of calcium, magnesium and phosphorus in an elderly population using duplicate diet sampling vs food composition tables. *J Nutr Health Aging,* 5**:** 253-255, 2001
13. Oenning LL, Vogel J, Calvo MS: Accuracy of methods estimating calcium and phosphorus intake in daily diets. *J Am Diet Assoc,* 88**:** 1076-1080, 1988
14. Sherman RA, Mehta O: Dietary phosphorus restriction in dialysis patients: potential impact of processed meat, poultry, and fish products as protein sources. *Am J Kidney Dis,* 54**:** 18-23, 2009
15. Sullivan CM, Leon JB, Sehgal AR: Phosphorus-containing food additives and the accuracy of nutrient databases: implications for renal patients. *J Ren Nutr,* 17**:** 350-354, 2007
16. Vorland CJ, Martin BR, Weaver CM, Peacock M, Hill Gallant KM: Phosphorus balance in adolescent girls and the effect of supplemental dietary calcium. *JBMR Plus,* 1**:** XX-XX, 2017
17. Palomino HL, Rifkin DE, Anderson C, Criqui MH, Whooley MA, Ix JH: 24-hour urine phosphorus excretion and mortality and cardiovascular events. *Clin J Am Soc Nephrol,* 8**:** 1202-1210, 2013
18. Robinson-Cohen C, Ix JH, Smits G, Persky M, Chertow GM, Block GA, Kestenbaum BR: Estimation of 24-hour urine phosphorushosphate excretion from spot urine collection: development of a predictive equation. *J Ren Nutr,* 24**:** 194-199, 2014
19. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl***:** S1-130, 2009
20. Isakova T, Ix JH, Sprague SM, Raphael KL, Fried L, Gassman JJ, Raj D, Cheung AK, Kusek JW, Flessner MF, Wolf M, Block GA: Rationale and Approaches to Phosphate and Fibroblast Growth Factor 23 Reduction in CKD. *J Am Soc Nephrol,* 26**:** 2328-2339, 2015
21. Hill KM, Martin BR, Wastney ME, McCabe GP, Moe SM, Weaver CM, Peacock M: Oral calcium carbonate affects calcium but not phosphorus balance in stage 3-4 chronic kidney disease. *Kidney Int,* 83**:** 959-966, 2013
22. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D: A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med,* 130**:** 461-470, 1999

23. Weaver CM: Clinical Approaches for Studying Calcium Metabolism and Its Relationship to Disease. In: *Calcium in Human Health.* edited by WEAVER, C. M., HEANEY, R. P., Humana Press, 2006, pp 65-81
24. Wilkinson R: Polyethylene glycol 4000 as a continuously administered non-absorbable faecal marker for metabolic balance studies in human subjects. *Gut,* 12**:** 654-660, 1971
25. Schiller LR, Santa Ana CA, Porter J, Fordtran JS: Validation of polyethylene glycol 3350 as a poorly absorbable marker for intestinal perfusion studies. *Dig Dis Sci,* 42**:** 1-5, 1997
26. Fleiss J: *The design and analysis of clinical experiments,* New York, NY, John Wiley & Sons, 1986
27. Lemann J: Calcium and phosphate metabolism: An overview in health and in calcium stone formers. In: *Kidney Stones: Medical and Surgical Management.* edited by COE, F., FAVUS, M., PAK, C., PARKS, J., PREMINGER, G., Philadelphia, PA, Lippincott-Raven Publishers, 1996, pp 259-288
28. Cho MM, Yi MM: Variability of daily creatinine excretion in healthy adults. *Hum Nutr Clin Nutr,* 40**:** 469-472, 1986
29. Edwards OM, Bayliss RI, Millen S: Urinary creatinine excretion as an index of the copleteness of 24-hour urine collections. *Lancet,* 2**:** 1165-1166, 1969
30. Waterlow JC: Observations on the variability of creatinine excretion. *Hum Nutr Clin Nutr,* 40**:** 125-129, 1986
31. Greenblatt DJ, Ransil BJ, Harmatz JS, Smith TW, Duhme DW, Koch-Weser J: Variability of 24-hour urinary creatinine excretion by normal subjects. *J Clin Pharmacol,* 16**:** 321-328, 1976
32. Weaver CM, Martin BR, McCabe GP, McCabe LD, Woodward M, Anderson CA, Appel LJ: Individual variation in urinary sodium excretion among adolescent girls on a fixed intake. *J Hypertens,* 34**:** 1290-1297, 2016
33. Agarwal R: Reproducibility of renal function measurements in adult men with diabetic nephropathy: research and clinical implications. *Am J Nephrol,* 27**:** 92-100, 2007
34. Hsu CY, Propert K, Xie D, Hamm L, He J, Miller E, Ojo A, Shlipak M, Teal V, Townsend R, Weir M, Wilson J, Feldman H, Investigators C: Measured GFR does not outperform estimated GFR in predicting CKD-related complications. *J Am Soc Nephrol,* 22**:** 1931-1937, 2011

35. Nafz B, Ehmke H, Wagner CD, Kirchheim HR, Persson PB: Blood pressure variability and urine flow in the conscious dog. *Am J Physiol,* 274**:** F680-686, 1998
36. Sakuma M, Morimoto Y, Suzuki Y, Suzuki A, Noda S, Nishino K, Ando S, Ishikawa M, Arai H: Availability of 24-h urine collection method on dietary phosphorus intake estimation. *J Clin Biochem Nutr,* 60**:** 125-129, 2017
37. Rakova N, Kitada K, Lerchl K, Dahlmann A, Birukov A, Daub S, Kopp C, Pedchenko T, Zhang Y, Beck L, Johannes B, Marton A, Muller DN, Rauh M, Luft FC, Titze J: Increased salt consumption induces body water conservation and decreases fluid intake. *J Clin Invest,* 127**:** 1932-1943, 2017
38. Moe SM, Zidehsarai MP, Chambers MA, Jackman LA, Radcliffe JS, Trevino LL, Donahue SE, Asplin JR: Vegetarian compared with meat dietary phosphorusrotein source and phosphorus homeostasis in chronic kidney disease. *Clin J Am Soc Nephrol,* 6**:** 257-264, 2011
39. Scanni R, vonRotz M, Jehle S, Hulter HN, Krapf R: The human response to acute enteral and parenteral phosphate loads. *J Am Soc Nephrol,* 25**:** 2730-2739, 2014
40. Turner ME, White CA, Hopman WM, Ward EC, Jeronimo PS, Adams MA, Holden RM: Impaired Phosphate Tolerance Revealed With an Acute Oral Challenge. *J Bone Miner Res,* 33**:** 113-122, 2018
41. Selamet U, Tighiouart H, Sarnak MJ, Beck G, Levey AS, Block G, Ix JH: Relationship of dietary phosphorushosphate intake with risk of end-stage renal disease and mortality in chronic kidney disease stages 3-5: The Modification of Diet in Renal Disease Study. *Kidney Int,* 89**:** 176-184, 2016
42. Spiegel DM, Brady K: Calcium balance in normal individuals and in patients with chronic kidney disease on low- and high-calcium diets. *Kidney international,* 81**:** 1116-1122, 2012
43. Hill Gallant KM, Spiegel DM: Calcium Balance in Chronic Kidney Disease. *Curr Osteoporos Rep,* 15**:** 214-221, 2017
44. Rothfuss KS, Bode JC, Stange EF, Parlesak A: Urinary excretion of polyethylene glycol 3350 during colonoscopy preparation. *Z Gastroenterol,* 44**:** 167-172, 2006
45. Block GA, Wheeler DC, Persky MS, Kestenbaum B, Ketteler M, Spiegel DM, Allison MA, Asplin J, Smits G, Hoofnagle AN, Kooienga L, Thadhani R, Mannstadt M, Wolf M, Chertow GM: Effects of phosphate binders in moderate CKD. *J Am Soc Nephrol,* 23**:** 1407-1415, 2012

46. Isakova T, Gutierrez OM, Smith K, Epstein M, Keating LK, Juppner H, Wolf M: Pilot study of dietary phosphorus restriction and phosphorus binders to target fibroblast growth factor 23 in patients with chronic kidney disease. *Nephrol Dial Transplant,* 26**:** 584-591, 2011
47. Moorthi RN, Armstrong CL, Janda K, Ponsler-Sipes K, Asplin JR, Moe SM: The effect of a diet containing 70% protein from plants on mineral metabolism and musculoskeletal health in chronic kidney disease. *Am J Nephrol,* 40**:** 582-591, 2014

Supplemental Information

Supplemental Methods

Recruitment, Inclusion/Exclusion Criteria, and Enrollment Dates

For the parent study, patients were recruited from nephrology clinics. Inclusion criteria included 1) men or women 35 years of age or older of any race/ethnicity, 2) GFR ≤ 45 mL/min, 3) intact serum PTH ≥ 37 pg/mL (above the mean of the normal range), 4) ability to perform two 3-week balance studies, 5) on stable doses of diuretics for at least two months, and 6) women were required to be post-menopausal (21). Exclusion criteria included 1) uncontrolled underlying disease (e.g. diabetes, lupus, hypertension), 2) taking drugs that alter calcium and phosphorus homeostasis including cholecalciferol (besides that given as part of the study protocol), ergocalciferol, active vitamin D metabolites, calcimimetics, or PTH analogues in the prior 30 days, 3) plans to initiate dialysis within 6 months, 4) serum calcium > 10.5 mg/dL, 5) serum phosphorus > 5.5 mg/dL, 6) intestinal disease that alters mineral absorption (e.g. celiac disease, small bowel resection, bariatric surgery). As previously reported (21), one patient inadvertently remained on 2 mcg/d paricalcitol during the study, but, despite this, had the lowest calcium and phosphorus absorption values. Rolling enrollment began in May 2010 and continued through August 2011, with the final subject completing the study in November 2011.

Adherence Assurance Measures

Adherence assurance measures were taken during the balance studies. Pill counts, diet checklists, encouragement at each meal to consume all food and beverages, and leftover weigh-back recording and analysis of leftovers for mineral content were used for adherence with diet and supplements. A non-absorbable fecal marker (polyethylene glycol, PEG m.w.3500) was given 3/g day to assess fecal collection adherence, and urine Cr was measured for each day to account for urine collection adherence. Research center staff were trained to appropriately time, collect, record, and measure urine volume from collections. Steps were taken to minimize error in urine collection timing and pooling: the start and end time of each collection was written on the urine collection bottle kept in the patient room; urine collection end times were designated on the study visit flowsheets and overseen by research center staff; at the end time of the collection, the

completed urine collection bottle was removed from the patient room; and a set of dedicated graduated cylinders were used to measure urine volumes of collections to the nearest 1 mL. As previously reported(21), dietary adherence was high (>90% of prescribed dietary calcium and phosphorus consumed), which was assessed by weighing back any uneaten portions of food items and analyzing for phosphorus content, and subtracting from the full cycle menu day meal values. Creatinine-corrected 24-hour urine phosphorus values differed minimally from uncorrected values (0.0-27 mg/d); adherence for calcium carbonate and placebo was 100% by pill count; and mean percent fecal PEG recovery was >80%.

Statistical Methods: Standard Deviation, %CV, and Reliability Calculations

Within-Subject Variation. Within-subject standard deviations (s_i where i = subject 1 to 8) were calculated from the individual 13 collections ($x_{i,j}$ where j = collection 1 to 13) and the mean of the 13 collections within that subject ($\bar{x}_i$): $s_i = \sqrt{\frac{\sum(x_{i,j}-\bar{x}_i)^2}{13-1}}$. Within-subject %CV ($\%CV_i$) was calculated from these within-subject standard deviations (s_i) and the within-subject mean of 13 collections ($\bar{x}_i$): $\%CV_i = \frac{s_i}{\bar{x}_i} \cdot 100$.

Among-Subject Variation. Among-subject standard deviations (S) were calculated from each subject's 13-day average ($\bar{x}_i$), and the mean of all eight subjects' 13-day averages ($\bar{X}$): $S = \sqrt{\frac{\sum(\bar{x}_i-\bar{X})^2}{8-1}}$. Among-subject %CV was calculated from this among-subject standard deviation and the among-subject mean of 13-day averages: $\%CV = \frac{S}{\bar{X}} \cdot 100$.

Reliability (Spearman-Brown Prediction Formula). The Spearman-Brown Prediction Formation is $r^* = n^*rho/(1+(n-1)rho)$, where *rho* is the reliability of the reliability of a single measure (intraclass correlation coefficient), and r^* is the reliability of the measure with n number of replicates(26).

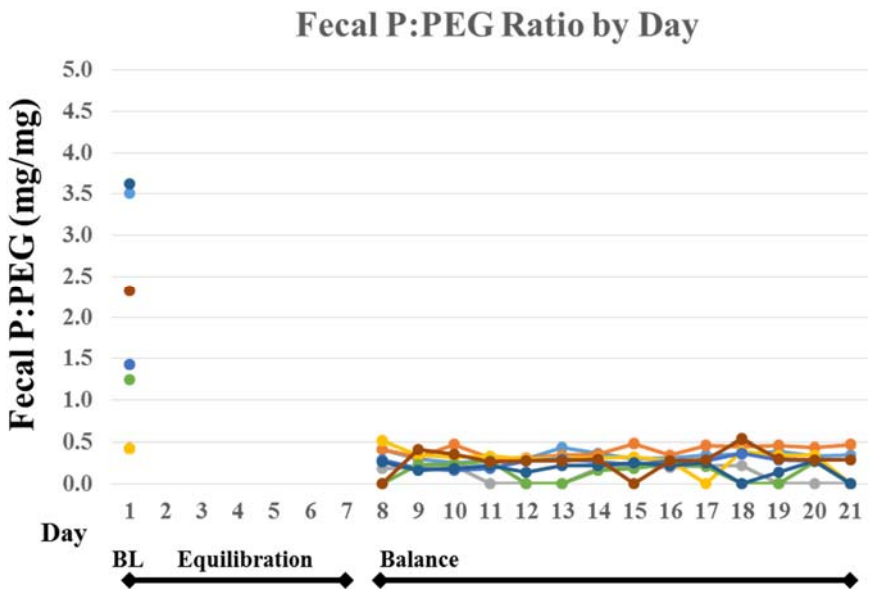
Supplemental Results

Variation and Reliability of Fecal P, Ca, and P:PEG and Ca:PEG Ratios

As expected, very wide within and among subject variation in absolute values (mg) of daily fecal P and Ca excretion were observed. For fecal P, standard deviations within subjects averaged 449 mg/d and ranged from 301 to 687 mg/d (%CV=54, range 45-62%) and among subjects

standard deviation was 193 mg/d (%CV = 24%). For fecal Ca, standard deviations within subjects averaged 533 mg/d and ranged from 301 to 1054 mg/d (%CV=53, range 46-62%) and among subjects standard deviation was 400 mg/d (%CV = 40%). Adjustment for fecal PEG excretion by expressing fecal P and Ca as P:PEG and Ca:PEG (mg:mg) ratios greatly reduced within subject variation: for fecal P, within subject standard deviations averaged 0.053 and ranged from 0.015 to 0.083 mg:mg (%CV = 18, range 8-26%), and for fecal Ca:PEG, within subject standard deviations averaged 0.048 and ranged from 0.021 to 0.067 mg:mg (%CV=15, range 8-21%). Among subjects, fecal P:PEG standard deviation was 0.072 mg:mg (%CV=25%) and fecal Ca:PEG standard deviation was 0.040 mg:mg (%CV=12%). The intraclass correlation coefficient for fecal P (unadjusted mg) was rho = 0.08 (95%CI: 0, 0.38), for fecal Ca (unadjusted mg) was rho= 0.19 (95%CI: 0.05, 0.55), for fecal P:PEG ratio was rho = 0.60 (95%CI: 0.37, 0.87), and for fecal Ca:PEG ratio was rho= 0.36 (95%CI: 0.16 , 0.72). From these values, the number of replicates needed to achieve ≥75% reliability for fecal P is 33 (95%CI: 6, unable to define upper-bound), for fecal Ca is 14 (95%CI: 3, 56), for fecal P:PEG ratio is 2 (95%CI: 1, 6), and for fecal Ca:PEG ratio is 6 (95%CI: 2, 16).

A)



B)

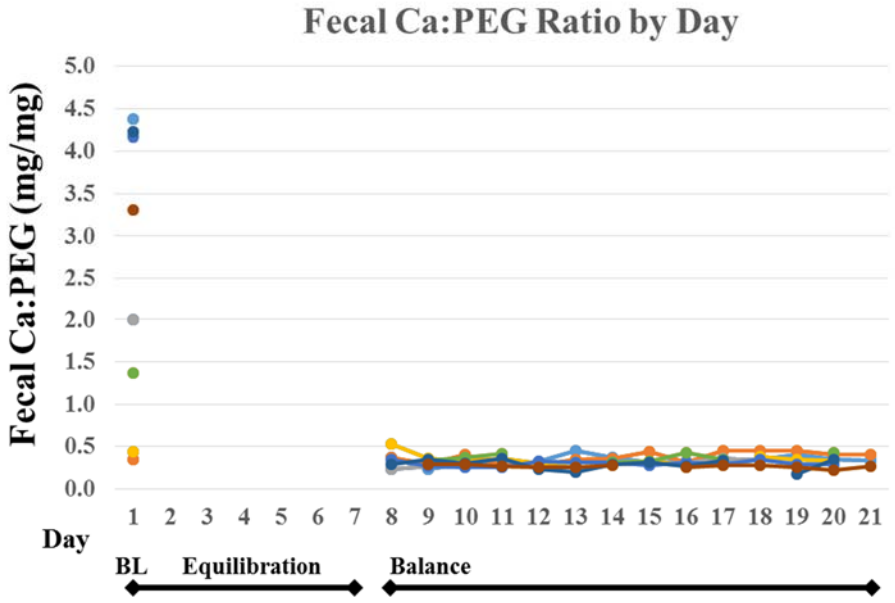


Figure S1. Daily variation in **A**) Fecal P:PEG ratios and **B**) Fecal Ca:PEG ratios during the balance studies. Day 1-7 is the diet equilibration period, and Day 8-21 is the balance period. BL = baseline, which represents a fecal collection before the controlled diet began and before the 3 g/d of PEG were given.

CHAPTER 3: INTESTINAL PHOSPHORUS ABSORPTION IN MODERATE CKD AND HEALTHY ADULTS USING A RADIOISOTOPIC TRACER FOR DIRECT MEASURES OF ABSORPTION

Abstract

Background and objectives Intestinal phosphorus absorption in patients with CKD is understudied, yet current therapies focus on reducing intestinal phosphorus absorption to lower the risk of cardiovascular dysfunction and mortality. Rodent studies suggest that intestinal phosphorus absorption remains inappropriately normal in early CKD, despite elevations in fibroblast growth factor 23 (FGF23) and declining 1,25-dihydroxycitamin D (1,25D). We have previously shown that 24h urine phosphorus is not related to net phosphorus absorption from metabolic balance studies in CKD patients (Stremke et al. CJASN 2018), and thus more direct measures of absorption are needed.

Design, setting, participants, &measurements In this controlled feeding study, we aimed to determine intestinal phosphorus absorption in patients with moderate CKD vs healthy subjects using a phosphorus (^{33}P) radioisotope tracer method. CKD and controls were matched for age (+/- 10y), sex, and race. Subjects ate a controlled study diet of ~1500 mg/d P, ~1400 mg/d Ca, ~3200 mg/d K, ~2400 mg/d Na, and 0.8 g/kg/d protein for 8 days. The final two study days consisted of an inpatient clinical research center (CRC) phosphorus absorption test utilizing oral and IV doses of ^{33}P. Fractional phosphorus absorption was determined by multi-compartment kinetic modeling. Fasting biochemistries and a 2-day average 24h urine phosphorus (uP) were determined. Differences between CKD and controls were determined by paired statistical analyses. The relationship between fractional phosphorus absorption and 24h uP was determined by Pearson correlation.

Results N=8 CKD patients (eGFR=29-55 mL/min/1.73m^2) and N=8 matched healthy controls completed the study. Fractional phosphorus absorption was similar between CKD and healthy controls (0.69 vs 0.62, p=0.52). 24h uP also did not differ (884 vs 935 mg/d, p=0.70). 24h uP did not relate to fractional phosphorus absorption overall (r=0.10, p=0.63), nor within either group (CKD, r= 0.15; healthy controls, r=0.13; p>0.50). CKD patients had higher serum intact parathyroid hormone (iPTH) (110 pg/mL vs 46 pg/mL; P=0.001) and intact FGF23 (89 pg/mL vs

34 pg/mL vs; P=0.005). Serum 1,25D values were significantly different between groups with 26 pg/mL and 38 pg/mL for CKD and healthy controls, respectively (P=0.03). There was no relationship between 1,25D and fractional phosphorus absorption (r=-0.16; P=0.56), despite significantly different mean serum 1,25D between groups.

Conclusions Fractional intestinal phosphorus absorption is similar between moderate CKD patients and healthy subjects consuming a controlled diet. This supports animal data that intestinal phosphorus absorption is maintained at inappropriately normal levels in early/moderate CKD despite hormonal changes that should suppress phosphorus absorption. We also show no association between CRC collected 24h uP and fractional phosphorus absorption.

Introduction

Reducing intestinal phosphorus absorption is the focus of therapies for management and prevention of chronic kidney disease-mineral bone disorder (CKD-MBD) (1). These therapies include dietary phosphorus restriction and/or phosphate binding medications (2-4)), along with numerous pharmaceuticals in development targeting intestinal phosphorus absorption (5, 6). However, intestinal phosphorus absorption, and mechanisms controlling phosphorus absorption, is understudied.

There are few *in vivo* intestinal phosphorus absorption studies in animals (7-9) or human (10, 11), in either health or CKD. The effect of moderate CKD on intestinal phosphorus absorption is unclear, but the hormonal changes (i.e. decreased 1,25-dihydroxyvitamin D) observed at these stages (12) would suggest that intestinal phosphorus absorption should be lower in these patients compared to healthy persons. This is due to the relationship between 1,25D and its regulation of intestinal phosphate transporters previously established *in vitro* (13, 14).

We (15) and others (16) have shown that intestinal phosphorus absorption is not lower in rat models of CKD using *in vivo* absorption assessment methods, despite reduced 1,25-dihydroxyvitamin D (1,25D). This has not been rigorously tested in human studies of patients with moderate CKD. We have recently shown (17) that 24-hour urine phosphorus (24h uP) is not a reliable biomarker of intestinal phosphorus absorption in moderate CKD patients in a secondary analysis of a controlled feeding metabolic balance study (18). Together, these data indicate the need for more direct assessment measures of intestinal phosphorus absorption.

The use of isotopic tracers to measure intestinal absorption of minerals has been considered the gold-standard. (19, 10, 11). This is due to the ability to define mineral transport rates between physiological compartments by using direct measures of known doses of isotopes (20). However, no stables isotopes of phosphorus exist (beyond the abundant ^{31}P), and there are only two useful radioisotopes: ^{32}P (a high energy β particle emitter) and ^{33}P (a low energy β particle emitter). Previous studies utilizing phosphorus radioisotopes have been in patients in end-stage renal disease (ESRD), and have used ^{33}P together with the high energy ^{32}P, presenting more hazardous radiation dose to the subjects. Therefore, the aim of this study was to determine fractional intestinal phosphorus absorption in patients with moderate CKD compared to healthy adults via a isotope absorption test protocol using a single isotope, ^{33}P, in a protocol that mimics the gold-standard dual isotope absorption test.

Methods

Study Design and Participants

This study was a parallel-arm study designed to assess fractional intestinal phosphorus absorption between moderate CKD patients and healthy adults. Inclusion criteria for moderate CKD subjects included men and women between aged 30-75 years with estimated glomerular filtration rate (eGFR) 45-59 ml/min/1.73 m^2 (stage 3a CKD) with A2 or A3 albuminuria or 30-44 ml/min/1.73 m^2 (stage 3b CKD) with or without albuminuria. Participants were required to be stable on all medications for six weeks prior to study enrollment and were required to discontinue all medications or nutritional supplements that could alter phosphorus metabolism. Healthy control subjects where recruited using a voluntary database maintained via the Indiana Clinical Translational Science Institute (Indiana CTSI) titled 'INResearch.' Healthy controls were race-, sex-, and age-matched (+/- 10 years) to CKD patients. Healthy participants were required to have normal kidney function as assessed by a nephrologist and no presence of albuminuria. Healthy controls were required to have normal fasting serum calcium, phosphorus, and parathyroid hormone (PTH) values. N=8 moderate CKD patients and N=8 healthy matched controls completed the study.

Enrolled subjects participated in a 9-day controlled-feeding study. For Days 1-6, subjects consumed a controlled diet as outpatients and on day 7 were admitted to the Indiana Clinical

Research Center (CRC) in Indianapolis, IN as inpatients for two days for phosphorus absorption testing (**Figure 3.1**.). All study protocols were approved by the Indiana University Institutional Review Board (IRB Protocol Number 1612460566). This study was registered with Clinicaltrials.gov (NCT03108222).

Controlled Diet, Dietary Nutrient Analysis, and Compliance

The study diet consisted of a 3-day cycle menu designed by a registered dietitian bionutritionist using ProNutra (Viocare, Inc., Princeton, NJ) dietary analysis software. All study meals, outpatient pack-outs and inpatient meals, were prepared in a metabolic kitchen where raw ingredients were measured for accuracy to the 0.1 gram. The cycle menu was designed as a high-phosphorus diet, reflective of the average US daily phosphorus intake (21, 22) and was designed to contain approximately 1500 mg of phosphorus. Energy, protein (0.8 g/kg/day), fiber and mineral (~1400 mg/d calcium, ~2400 mg/d sodium, and ~3200 mg/d potassium) composition was also controlled during the study period. Average kilocalories (kcal) computed from ProNutra were 2240 ± 15 kcal per day. The diet was designed to be consistent in nutrient content across the 3 day cycle menu.

Actual nutrient composition of daily diet composites the 3-day cycle menu was determined by Inductively coupled plasma – optical emission spectrometry (ICP-OES). Actual dietary compliance during the inpatient stay was assessed using weights from leftover food after meals and dietary components were calculated as percent of meal consumed.

Fractional Phosphorus Absorption and Phosphorus Kinetics

The inpatient phosphorus absorption test included oral and IV doses of ^{33}P. On study day 7, a fasting blood sample and urine sample were taken upon admission to the CRC. Following fasting samples, an oral dose of 10 μCi of ^{33}P as orthophosphoric acid (American Radiolabeled Chemicals, In.; St. Louis, MO) in 120 mL mineral-free water was administered to the patient halfway through the first meal of the day (breakfast). After the oral dose was administered, patients finished the remainder of their meal. Serial blood draws then occurred at the following time points: 15, 30, 45, 60, 90 minutes and 2, 3, 4, 6, and 24 hours. Urine samples were pooled and collected during the following intervals post-dosing: 0-2 hours (h) post-dose, 2-4h post-dose; 4-6h post-

dose; 6-24h post-dose; and 24-25h post-dose. All inpatient stool samples were collected and times recorded. At 25h post-oral dose, a new baseline blood draw was taken, then subjects were administered an IV dose of 10 uCi of ^{33}P in sterile saline. Serial blood draws, urine and fecal collections post-IV dose followed the same protocol as oral dosing.

^{33}P activity in serum, urine, and feces was measured via liquid scintillation counting (Tri-Carb 2910 TR Liquid Scintillation Analyzer, Perkin Elmer, Waltham, MA). The primary outcome, fractional phosphorus absorption, was determined by multi-compartment kinetic modeling using general equation solving software (WINSAAM) and represents the fraction of the tracer entering circulation relative to the total dose moving through the digestive system. Details on the compartmental model are provided in the supplemental material.

Serum Biochemical Measures

Fasting biochemistries (serum calcium, phosphorus, creatinine, and eGFR) at screening and study Day 1 were analyzed by the Indiana University Pathology Laboratories. Inpatient blood and urine samples were analyzed by RX Daytona Clinical Chemistry Analyzer (Randox Laboratories Ltd, Crumlin, United Kingdom). Serum 1,25D was measured via liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Mayo Clinic Laboratories, Rochester, MN). Serum PTH was measured using Intact PTH (iPTH) 1-84 ELISA (Alpco, Salem, NH). Serum FGF23 was measured using MedFronteir Intact FGF23 (iFGF23) CLEIA (Eagle Biosciences, Amherst, NH).

Phosphorus Balance, Urine Phosphorus, and Fecal Phosphorus

A 9-day controlled feeding study was designed based on evidence of changes in phosphorus metabolism after one week of controlled feeding (23) and previous studies showing one week of controlled feeding was adequate to enter steady-state for calcium metabolism (24, 25, 18). All subjects consumed pre-prepared pack-out meals for study days 1-6. On study day 7, subjects were admitted to the CRC for a 48-hour inpatient stay. During the inpatient study, all feces and urine was collected. Urine samples were acidified with 1% (volume/volume) concentrated hydrogen chloride (HCl) at the time of aliquoting, and diluted with 2% nitric acid for mineral analysis by ICP-OES. Fecal samples were homogenized, aliquoted into microwave vessels

with 5 mL of 70% nitric acid and 5ml of ultrapure water. Fecal samples were digested in microwave (MARS 6, CEM, Matthews, NC) at 210 C for 35 minutes. Digested fecal samples were diluted with ultrapure water to 2% nitric acid for mineral analysis (Optima 4300DV, Perkin Elmer, Shelton, CT) (18). Mineral content of feces was adjusted based on excretion of a non-absorbable fecal maker over 48-hour dose, polyethylene-glycol (PEG), to account for loss of sample during collection and fecal transit time. 2.96 ± 0.12 g/day of PEG was taken with each meal during the study period. PEG concentration of fecal samples was measured via turbidimetric assay (26).

Phosphorus balance was calculated using 2-day average values for the dietary phosphorus intake as an inpatient, urinary excretion of phosphorus, and fecal phosphorus excretion (PEG-adjusted) (17). Phosphorus balance values were derived from the following equation: Phosphorus Balance = Dietary phosphorus intake – urinary phosphorus excretion – fecal phosphorus excretion.

Statistical Analyses

All statistical analyses were performed using SAS v9.4 (SAS Institute, Inc., Cary, NC) with statistical significance set at $\alpha=0.05$. Descriptive statistics were performed for subject demographics, baseline biochemical measures, and dietary compliance. Linear regression (PROC GLM) was used to determine differences between CKD and controls for the outcomes of fractional phosphorus absorption, absolute phosphorus absorption, 24-hour urine phosphorus, phosphorus balance, and serum 1,25D, iPTH, iFGF23, taking matched pairs into account in the regression model. The relationship between phosphorus absorption (fractional and absolute) and 24-hour urine phosphorus, and fractional phosphorus absorption and serum 1,25D was determined by Pearson correlation. All correlations were performed in all patients and within each group (CKD and healthy).

Power calculations to show equivalence indicated a total sample size of N=16 (N=8 per group) to provide 80% power to detect a fractional intestinal phosphorus absorption difference of 0.13 between groups with a standard deviation of 0.10 (27).

Results

Dietary Analysis and Subject Characteristics

Average dietary macronutrient (as calculated from ProNutra software) and mineral composition for the controlled diet, as prescribed is described in **Table 3.1**. The average value of phosphorus per day was 1828 mg/day. All mineral data presented from this study is based off of ICP mineral analysis. Mineral composition values of the prescribed diet differed between dietary software analysis and ICP analysis. These data can be found in supplemental table S1.

Subject characteristics for each study group are described in **Table 3.2**. BUN and eGFR were significantly different between the healthy controls and CKD patients, as expected by design. No other differences in baseline subject characteristics were observed.

Intestinal Phosphorus Absorption

There was no statistical difference in averages values for fractional intestinal phosphorous absorption or absolute intestinal phosphorus absorption between CKD and healthy controls. **Figure 3.2A** and **3.2B** show fractional intestinal phosphorus absorption and absolute intestinal phosphorus absorption, respectively. Healthy individuals had a mean phosphorus absorption fraction of 0.62 ± 0.07 and CKD patients had a mean phosphorus absorption fraction of 0.69 ± 0.06 (P=0.52). Similarly, absolute (mg) intestinal absorption of phosphorus for healthy individuals and CKD patients were 1137 ± 127 mg phosphorus/day and 1100 ± 106 mg phosphorus/day, respectively, and not statistically different (P=0.66). Mean whole-body phosphorus retention (over a two-day period) was not different between CKD patients (522 ± 106 mg/d) compared to healthy controls (649 ± 76 mg/d, P=0.40).

Twenty-Four-Hour Urinary Excretion and Relationship with Phosphorus Absorption

Figure 3.3A describes the 24-hour urinary excretion of phosphorus (2-day averaged) by study group. Healthy controls excreted a mean 935 ± 134 mg P/day and CKD patients excreted 884 ±334 mg P/day. These values were not related to intestinal fractional phosphorus absorption by the radioisotopic absorption method between groups (r=0.10; P=0.63) or within groups (CKD: r=0.17 and P=0.68; Healthy: r=0.13 and P=0.75) **Figure 3.3B**. **Figure 3.3C** also shows a non-significant relationship between 24h uP and the absolute (mg) amount of phosphorus absorbed in

the intestine (r =0.42 , P=0.11), though there is suggestion of a potential positive relationship. This relationship was also non-significant within study groups (CKD: r =0.43 P= 0.30; Healthy: r =0.52 , P = 0.18).

Phosphorus Regulatory Hormones

Figure 3.4A describes mean serum values for 1,25D, iPTH and iFGF23 of both study groups as least squared means values. CKD patients had lower mean 1,25D of 26 mg/mL compared with 38 pg/mL in healthy controls (P=0.03). Also as expected, CKD patients had higher serum iPTH compared to controls (110 ± 14 vs. 46 ± 5 pg/mL, respectively, P=0.001) as well as higher iFGF23 (89 ± 12 pg/mL vs 34 ± 3 pg/mL, respectively, P = 0.005).

Figure 3.4B shows a lack of relationship between serum 1,25D and fractional intestinal phosphorus absorption(r=-0.16; P=0.56), despite a significantly lower mean serum 1,25D in CKD compared with controls.

Discussion

Our results show fractional intestinal phosphorus absorption is similar between moderate CKD patients and healthy control subjects matched for age, sex, and race and consuming a controlled diet. But, CKD patients had lower serum 1,25D and higher iPTH and iFGF23, which is consistent with previously described biochemical alterations with kidney function decline (12). This reveals that intestinal phosphorus absorption in moderate CKD is inappropriately maintained at normal levels, despite changes in phosphorus regulatory hormones that should suppress absorption. Particularly, lower 1,25D should lead to decreased phosphorus absorption via suppressed sodium-dependent phosphate transport according to classic mechanistic understanding (14). The other main factor that is understood to influence intestinal phosphorus absorption efficiency (based largely on data from *in vitro/ex vivo* methods) is dietary phosphorus restriction, and this has been shown to be an effect that is independent of 1,25D (28). However, emerging data from *in vivo/in situ* assessments of intestinal phosphorus absorption are complicating our understanding of intestinal phosphorus absorption. We have recently shown using *in situ* intestinal ligated loop absorption methods that phosphorus absorption was not lower in CKD rats (Cy/+ model) compared to healthy normal rats, despite lower 1,25D levels in the CKD rats (15). This

matched the prior work by Marks et al. (16), who also showed no difference in intestinal phosphorus absorption by intestinal ligated loop method in 5/6 nephrectomized rats compared to sham-operated controls – again despite lower 1,25D in the nephrectomized rats. Our present study of intestinal phosphorus absorption efficiency in humans further supports these previous findings in two different CKD rat model. Further, in our previous rat study (15), we noted a peculiar finding – that fractional intestinal phosphorus absorption was slightly but statistically *higher* in CKD versus normal rats despite the lower 1,25D. Here, we again see (numerically but not statistically) slightly higher fractional phosphorus absorption in CKD patients compared with healthy controls. Marks and colleagues (16) also showed, numerically but non-significantly, slightly higher intestinal fractional absorption in the nephrectomized versus sham rats. These observations may well be spurious, but warrant further investigation of potential maladaptation of increasing intestinal phosphorus absorption in CKD in the context of declining 1,25D.

There are clinical implications for our findings that intestinal phosphorus absorption is maintained in moderate CKD patients at levels similar to healthy controls, even while 1,25D is reduced. First, phosphorus is absorbed at a relatively high rate in health, and this appears to persist in CKD. Notably the fractional phosphorus absorption values observed in our study are similar to those reported by Scanni and colleagues (29), who used enteral infusion of phosphate to determine absorption efficiency in healthy adults, which they reported as approximately 70%. The high rate of absorption that is maintained in CKD suggests that successfully blocking phosphorus absorption has the potential for a dramatic impact on phosphorus metabolism. Thus, there is strong rationale for continued efforts to develop better phosphate binders, absorption inhibitors, or other approaches to limit intestinal phosphorus absorption.

The use of a single ^{33}P radioisotope given orally and by IV to provides a direct measure of phosphorus absorption for use in clinical studies. Historically, radioisotope protocols for investigating intestinal phosphorus absorption have used both the high energy ^{32}P isotope and the lower energy ^{33}P isotope in a classic dual isotope method where both isotopes can be given concurrently (one orally and one by IV) and distinguished in the biological samples due to their different energy peaks by liquid scintillation counting (30, 19). ^{32}P, however, is a high energy beta particle emitter that results in a greater effective dose of radiation to the body's tissues compared with ^{33}P, so is potentially more hazardous. ^{33}P also has a research advantage due to the longer half-life (25.3 d) compared with ^{32}P (14.3 d), which makes it detectable in samples for longer periods

of time. Thus, we modified the classic dual isotope absorption method by given 33P as both the oral and IV dose, separated by 1 day, to take advantage of the longer half-life of ^{33}P and to reduce the effective dose of radiation to the patients. This is similar to the calcium absorption protocol we published previously using 45Ca isotope for both oral and IV doses separated by one day (18). The oral ^{33}P data is captured within the first 24 hours, then a new baseline is taken prior to the IV ^{33}P administration. The subsequent data through 48 hours is therefore regarded as clearance of the IV ^{33}P infusion for use in the multicompartment kinetic model to estimate fractional intestinal phosphorus absorption. Direct measures of intestinal phosphorus absorption, such as the dual isotope method or the modified single isotope method described here, are useful for physiological studies of intestinal phosphorus absorption in patients with CKD. We have previously reported that the traditional biomarker of phosphorus absorption, 24-hour urinary phosphorus excretion, may not be reflective of absorption in CKD patients (17). We retested this relationship in the current study and again show no significant association between 24-hour urinary phosphorus excretion and fractional phosphorus absorption. As we also stated in our previous study (17), we want to reiterate that these analyses do not preclude the use of 24h uP as a proxy of intestinal P absorption in the context of randomized controlled trials where the intervention has a known or suspected effect on absorption (e.g. phosphate binder trials). Rather, caution should be taken in interpretation of 24-hour urine phosphorus excretion from cross-sectional analyses or from intervention studies where there is no known or suspected effect on intestinal P absorption.

Strengths of this study included the controlled diet for 6 days prior to the phosphorus absorption testing, the inclusion of two 24-hour urine phosphorus measurements for a reliable value (17), inpatient phosphorus absorption testing using a single low-energy radioisotopic tracer to directly assess absorption by kinetic modeling, and the matching of patients to controls by race, sex, and age. A limitation of our study is the choice of a relatively high dietary phosphorus level, reflective of a typical American high phosphorus diet (21, 22). We chose this level as the most translational to real world patients. However, passive absorption plays a larger role when dietary phosphorus intakes are high (31, 32); thus, it is possible that a relationship between 1,25D status and intestinal phosphorus absorption would be detectable with a very low phosphorus intake. However, this notion is not supported by our previous rat study (8) that used a very low phosphorus concentration for the absorption testing and yielded similar conclusions.

In conclusion, our findings indicate that in moderate-stage CKD dietary phosphorus is absorbed at a level similar to healthy adults of similar age, sex, and race, and that there is not a detectable decrease in intestinal phosphorus absorption corresponding to lower 1,25D in these patients. These data underscore the need to development more effective strategies to address the high intestinal phosphorus absorption in patients with CKD.

Table 3.1 Average Daily Composition of Prescribed Diet. Data presented as mean (standard deviation).

Dietary Component	*Average value per day, as prescribed*
Energy (Kcal/day)	2240 (15)
Protein (g/day)	109 (10)
Carbohydrate (g/day)	288 (6)
Fat (g/day)	77 (3)
Phosphorus (mg/day)	1828 (101)
Calcium (mg/day)	884 (24)
Sodium (mg/day)	2487 (178)
Potassium (mg/day)	3121 (369)
Magnesium (mg/day)	332 (32)

Table 3.2 Subject demographics and baseline characteristics. Data presented as mean (SD). * indicates P value=0.008 and ** indicates P < 0.0001.

Baseline Characteristics	*Healthy (N=8)*	*CKD (N=8)*
Age, years	52 (13)	57 (14)
Female:Male, n	4:4	4:4
Black:White, n	3:5	3:5
BMI, kg/m^2	28.4 (4.7)	31.6 (8.7)
BUN, mg/dL	15(4)	32 (13)*
eGFR, mL/min/1.72m^2	88 (12)	41 (8)**
serum phosphorus, mg/dL	3.3 (0.4)	3.4 (0.6)
Serum Ca, mg/dL	9.6 (0.2	9.3 (0.3)

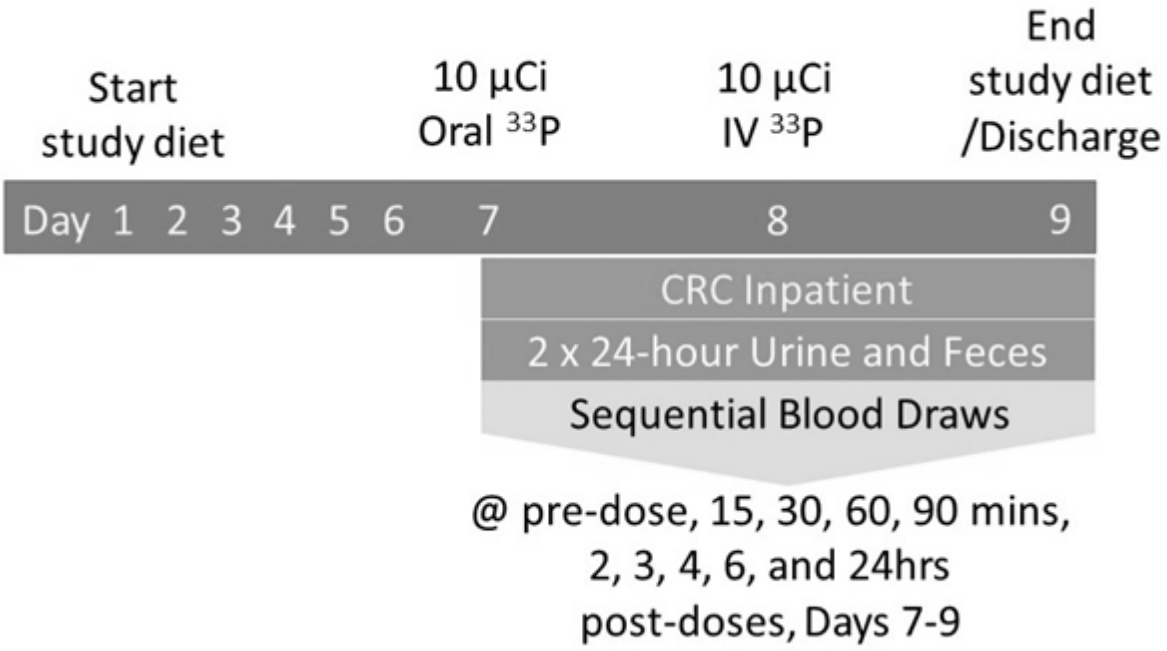
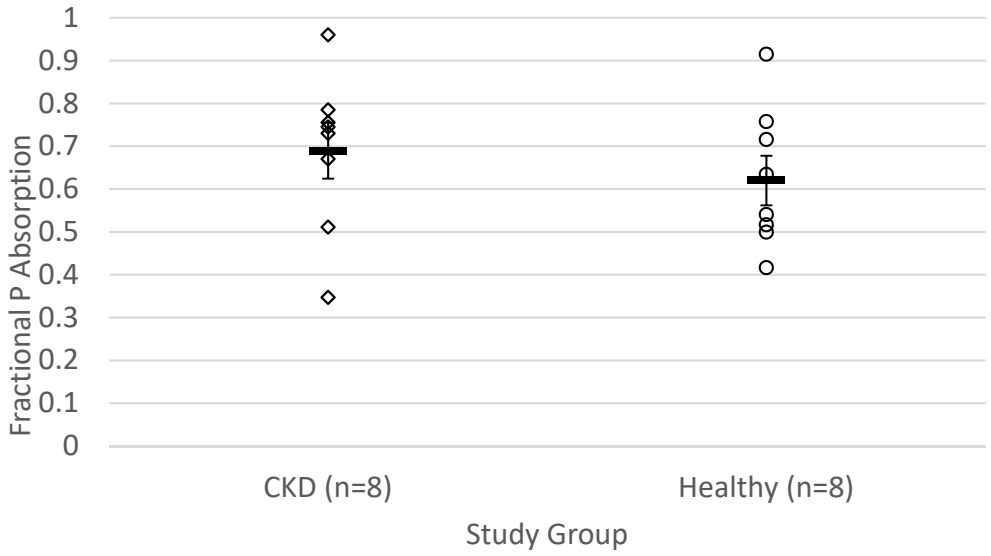


Figure 3.1 Intestinal Phosphorus Absorption Study Protocol

3.2A)



3.2B)

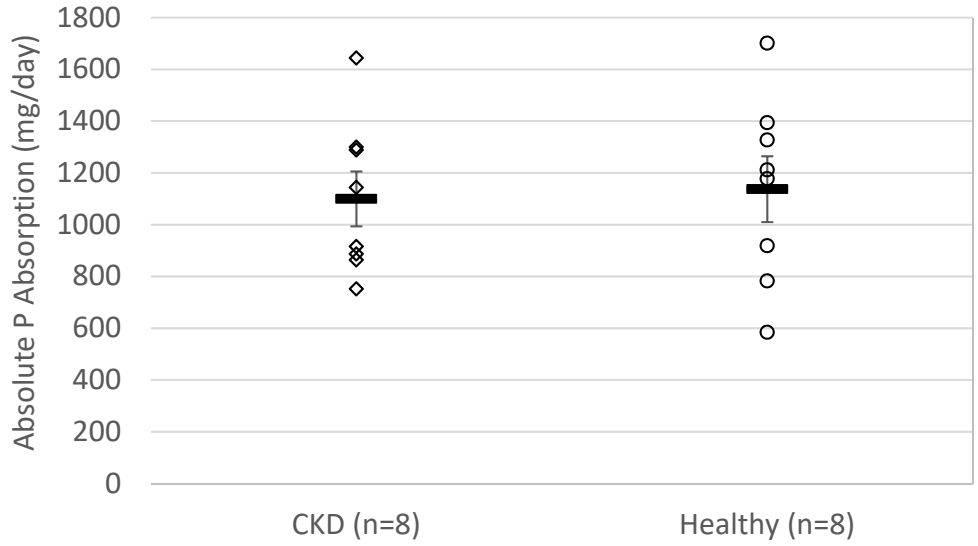
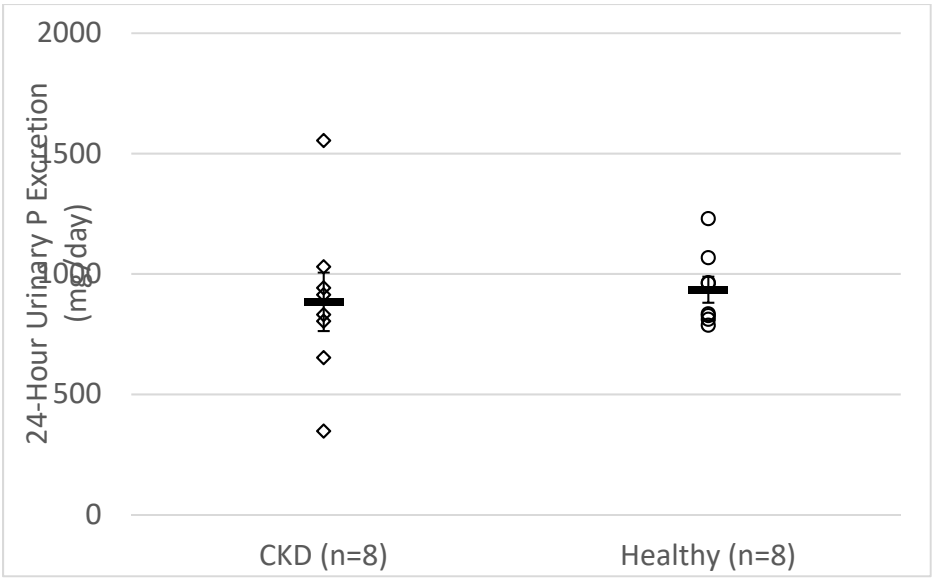


Figure 3.2 Phosphorus Absorption in Health and CKD. Bar graph showing A) Fractional Absorption in Health and CKD; and B) absolute P absorption in mg/day Health and CKD. Black lines indicate the means, error bars are SEM, and dots indicate values for individual subjects.

3.3A)



3.3B)

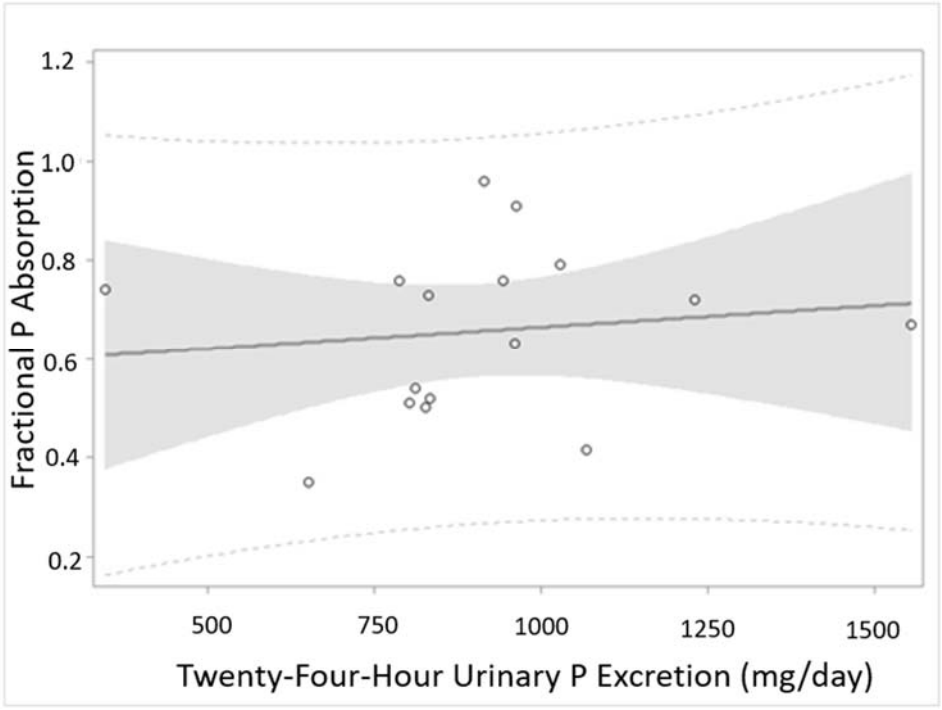
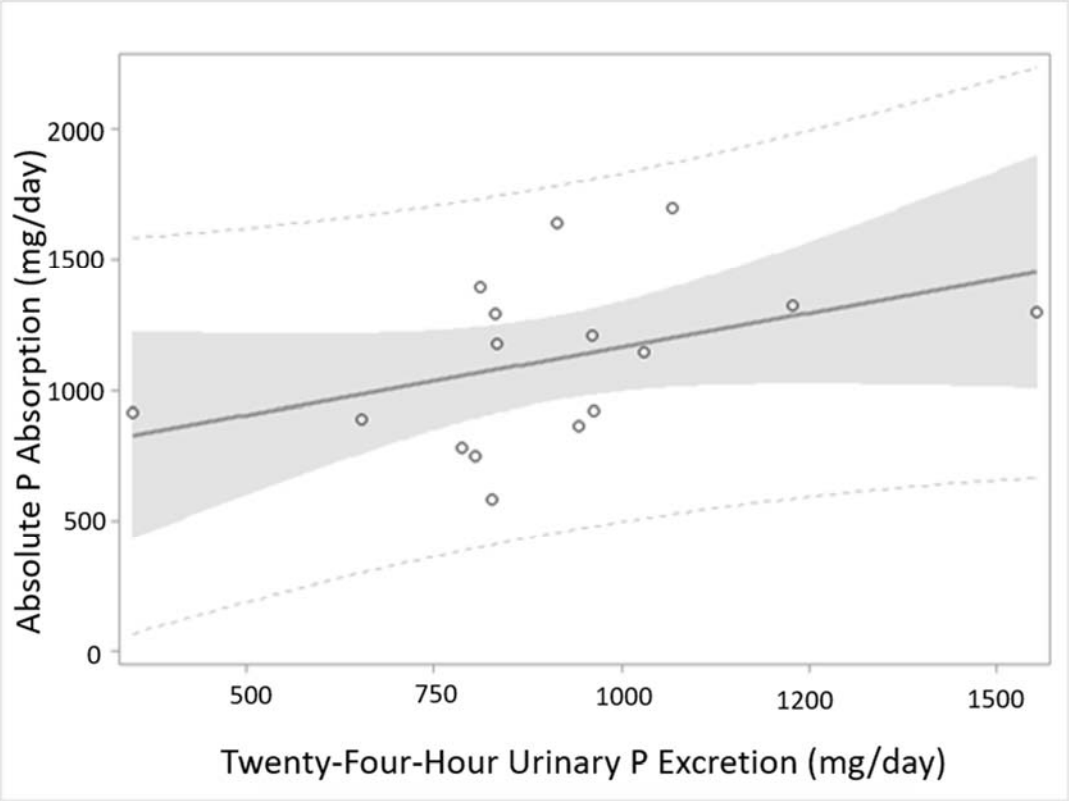


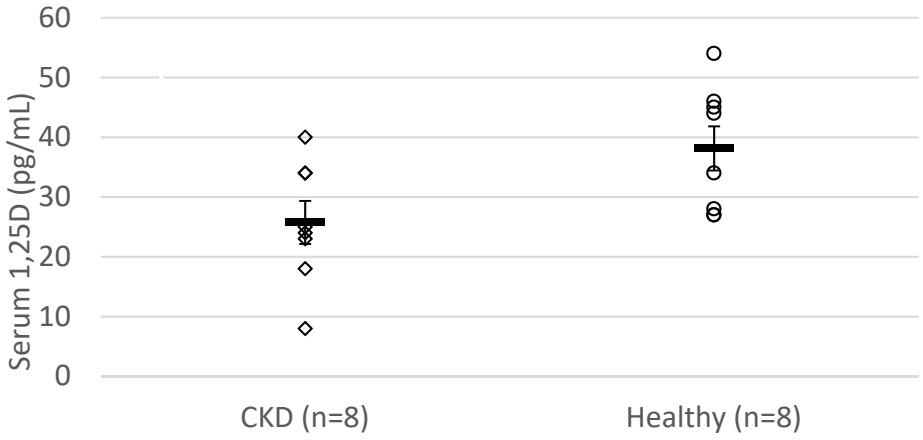
Figure 3.3 Twenty-Four Hour Urinary Phosphorus Excretion and its Relationship with Phosphorus Absorption. A) Twenty-four hour urine phosphorus excretion for each study group; B) The relationship between Twenty-four hour urine phosphorus excretion to fractional P absorption; and C) Absolute phosphorus absorption (mg/day). Black lines indicate the means, error bars are SEM, and dots indicate values for individual subjects. Solid line is the regression fit; shaded area is the 95% confidence limits; and the dotted lines indicate the 95% prediction limits.

Figure 3.3 continued

3.3C)



4.4A)



4.4B)

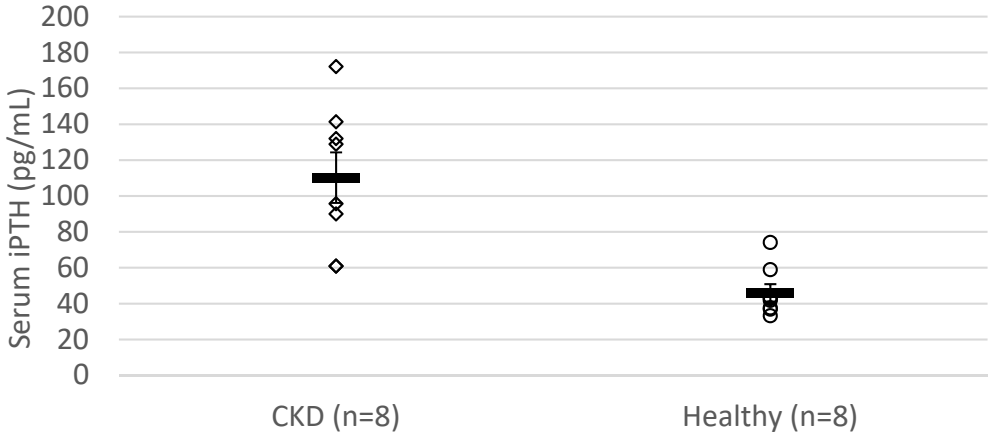
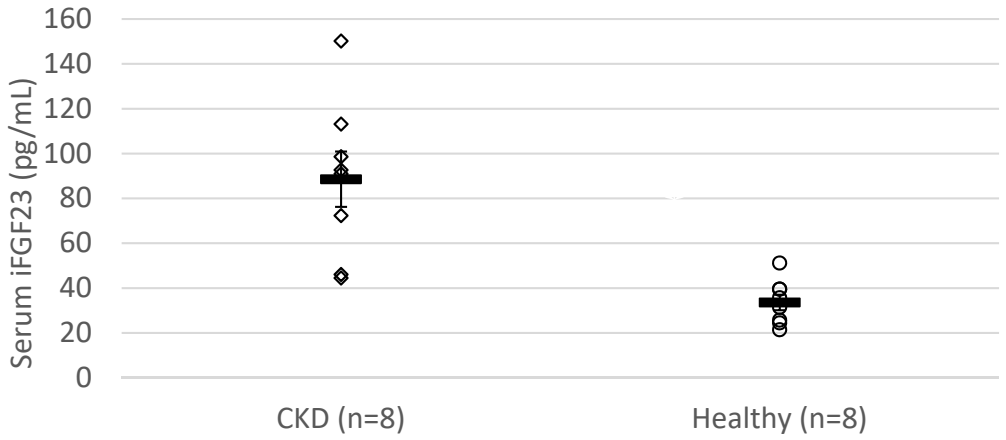


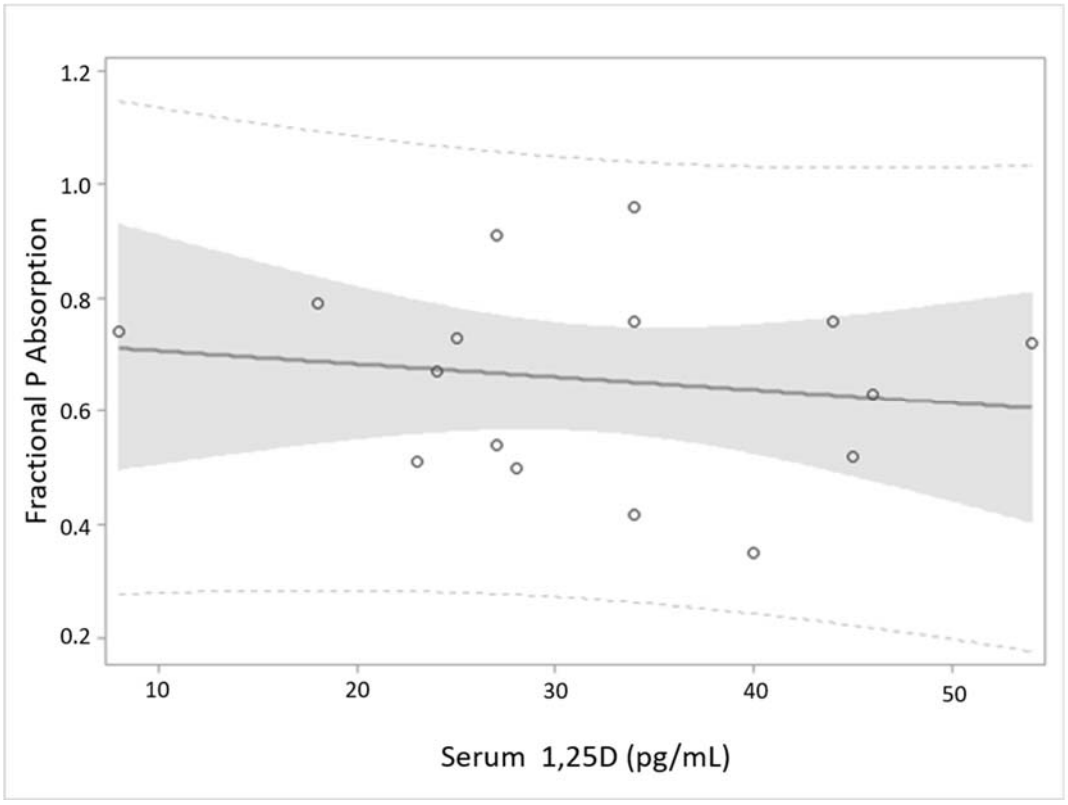
Figure 3.4 Serum Values of Phosphorus Regulatory Hormones. A) Serum 1,25D, B) iPTH, and C) iFGF23 in healthy controls and in moderate CKD. D) The relationship between 1,25D and fractional phosphorus absorption. Black lines indicate the means, error bars are SEM, and dots indicate values for individual subjects. Solid line is the regression fit; shaded area is the 95% confidence limits; and the dotted lines indicate the 95% prediction limits.

Figure 3.4 continued

4.4C)



4.4D)



References

1. Kidney Disease: Improving Global Outcomes CKDMBDUWG. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney Int Suppl (2011). 2017;7(1):1-59. doi:10.1016/j.kisu.2017.04.001.
2. Slatopolsky E, Rutherford WE, Rosenbaum R, Martin K, Hruska K. Hyperphosphatemia. Clin Nephrol. 1977;7(4):138-46.
3. Block GA, Wheeler DC, Persky MS, Kestenbaum B, Ketteler M, Spiegel DM et al. Effects of phosphate binders in moderate CKD. J Am Soc Nephrol. 2012;23(8):1407-15. doi:10.1681/ASN.2012030223.
4. Beto JA, Ramirez WE, Bansal VK. Medical nutrition therapy in adults with chronic kidney disease: integrating evidence and consensus into practice for the generalist registered dietitian nutritionist. J Acad Nutr Diet. 2014;114(7):1077-87. doi:10.1016/j.jand.2013.12.009.
5. Block GA, Rosenbaum DP, Yan A, Chertow GM. Efficacy and Safety of Tenapanor in Patients with Hyperphosphatemia Receiving Maintenance Hemodialysis: A Randomized Phase 3 Trial. J Am Soc Nephrol. 2019;30(4):641-52. doi:10.1681/ASN.2018080832.
6. Zhang Y, Ma T, Zhang P. Efficacy and safety of nicotinamide on phosphorus metabolism in hemodialysis patients: A systematic review and meta-analysis. Medicine (Baltimore). 2018;97(41):e12731. doi:10.1097/MD.0000000000012731.
7. Marks J, Lee GJ, Nadaraja SP, Debnam ES, Unwin RJ. Experimental and regional variations in Na+-dependent and Na+-independent phosphate transport along the rat small intestine and colon. Physiol Rep. 2015;3(1). doi:10.14814/phy2.12281.
8. Vorland CJ, Lachcik PJ, Aromeh LO, Moe SM, Chen NX, Hill Gallant KM. Effect of dietary phosphorus intake and age on intestinal phosphorus absorption efficiency and phosphorus balance in male rats. PLoS One. 2018;13(11):e0207601. doi:10.1371/journal.pone.0207601.
9. Turner ME, White CA, Hopman WM, Ward EC, Jeronimo PS, Adams MA et al. Impaired Phosphate Tolerance Revealed With an Acute Oral Challenge. J Bone Miner Res. 2018;33(1):113-22. doi:10.1002/jbmr.3294.

10. Farrington K, Mohammed MN, Newman SP, Varghese Z, Moorhead JF. Comparison of radioisotope methods for the measurement of phosphate absorption in normal subjects and in patients with chronic renal failure. Clin Sci (Lond). 1981;60(1):55-63. doi:10.1042/cs0600055.
11. Davis GR, Zerwekh JE, Parker TF, Krejs GJ, Pak CY, Fordtran JS. Absorption of phosphate in the jejunum of patients with chronic renal failure before and after correction of vitamin D deficiency. Gastroenterology. 1983;85(4):908-16.
12. Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. Kidney Int. 2011;79(12):1370-8. doi:10.1038/ki.2011.47.
13. Katai K, Miyamoto K, Kishida S, Segawa H, Nii T, Tanaka H et al. Regulation of intestinal Na+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D3. Biochem J. 1999;343 Pt 3:705-12.
14. Bergwitz C, Juppner H. Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. Annu Rev Med. 2010;61:91-104. doi:10.1146/annurev.med.051308.111339.
15. Vorland CJ, Biruete A, Lachcik PJ, Srinivasan S, Chen NX, Moe SM et al. Kidney Disease Progression Does Not Decrease Intestinal Phosphorus Absorption in a Rat Model of Chronic Kidney Disease-Mineral Bone Disorder. J Bone Miner Res. 2019. doi:10.1002/jbmr.3894.
16. Marks J, Churchill LJ, Srai SK, Biber J, Murer H, Jaeger P et al. Intestinal phosphate absorption in a model of chronic renal failure. Kidney Int. 2007;72(2):166-73. doi:10.1038/sj.ki.5002292.
17. Stremke ER, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM et al. Twenty-Four-Hour Urine phosphorus as a Biomarker of Dietary phosphorus Intake and Absorption in CKD: A Secondary Analysis from a Controlled Diet Balance Study. Clin J Am Soc Nephrol. 2018;13(7):1002-12. doi:10.2215/CJN.00390118.
18. Hill KM, Martin BR, Wastney ME, McCabe GP, Moe SM, Weaver CM et al. Oral calcium carbonate affects calcium but not phosphorus balance in stage 3-4 chronic kidney disease. Kidney Int. 2013;83(5):959-66. doi:10.1038/ki.2012.403.
19. Peacock M, Aaron JE, Walker GS, Davison AM. Bone disease and hyperparathyroidism in chronic renal failure: the effect of 1alpha-hydroxyvitamin D3. Clin Endocrinol (Oxf). 1977;7 Suppl:73s-81s. doi:10.1111/j.1365-2265.1977.tb03365.x.

20. Hart H, Spencer H. Rate of initial entry of Ca17 and Sr85 from the intestine into the vascular space. Proc Soc Exp Biol Med. 1967;126(2):365-71. doi:10.3181/00379727-126-32447.
21. Calvo MS, Uribarri J. Contributions to total phosphorus intake: all sources considered. Semin Dial. 2013;26(1):54-61. doi:10.1111/sdi.12042.
22. McClure ST, Chang AR, Selvin E, Rebholz CM, Appel LJ. Dietary Sources of Phosphorus among Adults in the United States: Results from NHANES 2001-2014. Nutrients. 2017;9(2). doi:10.3390/nu9020095.
23. Moorthi RN, Armstrong CL, Janda K, Ponsler-Sipes K, Asplin JR, Moe SM. The effect of a diet containing 70% protein from plants on mineral metabolism and musculoskeletal health in chronic kidney disease. Am J Nephrol. 2014;40(6):582-91. doi:10.1159/000371498.
24. Weaver CM, Rothwell AP, Wood KV. Measuring calcium absorption and utilization in humans. Curr Opin Clin Nutr Metab Care. 2006;9(5):568-74. doi:10.1097/01.mco.0000241666.46008.2c.
25. Spiegel DM, Brady K. Calcium balance in normal individuals and in patients with chronic kidney disease on low- and high-calcium diets. Kidney Int. 2012;81(11):1116-22. doi:10.1038/ki.2011.490.
26. DW MSP. An Improved Turbidimetric Analysis of Polyethylene Glycol Utilizing an Emulsifier. Gastroenterology. 1967;53(2):250-6. doi: https://doi.org/10.1016/S0016-5085(19)34232-5.
27. Chow SC, Shao J, Wang H. A note on sample size calculation for mean comparisons based on noncentral t-statistics. J Biopharm Stat. 2002;12(4):441-56. doi:10.1081/BIP-120016229.
28. Capuano P, Radanovic T, Wagner CA, Bacic D, Kato S, Uchiyama Y et al. Intestinal and renal adaptation to a low-Pi diet of type II NaPi cotransporters in vitamin D receptor- and 1alphaOHase-deficient mice. Am J Physiol Cell Physiol. 2005;288(2):C429-34. doi:10.1152/ajpcell.00331.2004.
29. Scanni R, vonRotz M, Jehle S, Hulter HN, Krapf R. The human response to acute enteral and parenteral phosphate loads. Journal of the American Society of Nephrology : JASN. 2014;25(12):2730-9. doi:10.1681/ASN.2013101076.
30. Nordin B. Calcium, Phosphate and Magnesium Metabolism. Edinburgh: Churchill Livingstone; 1976.

31. Lee DB, Walling MW, Brautbar N. Intestinal phosphate absorption: influence of vitamin D and non-vitamin D factors. Am J Physiol. 1986;250(3 Pt 1):G369-73. doi:10.1152/ajpgi.1986.250.3.G369.
32. Giral H, Caldas Y, Sutherland E, Wilson P, Breusegem S, Barry N et al. Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphorushosphate. Am J Physiol Renal Physiol. 2009;297(5):F1466-75. doi:10.1152/ajprenal.00279.2009.

Supplemental Information

Table S3.1. ProNutra Mineral Analysis compared to ICP-OES mineral analysis

Day	Phosphorus (mg/day)		Calcium (mg/day)		Sodium (mg/day)		Magnesium (mg/day)		Potassium (mg/day)	
	ProNutra	ICP	ProNutra	ICP	ProNutra	ICP	ProNutra	ICP	ProNutra	ICP
Cycle Day 1	1543	1807.17	1125	869.05	2306	2359.40	346	302.09	3016	2716.91
Cycle Day 2	1524	1937.57	1121	870.81	2307	2412.57	398	366.60	3049	3441.11
Cycle Day 3	1513	1739.13	1969	910.68	2713	2690.45	350	328.49	3511	3206.02
Average	1526.67	1827.95	1405	883.51	2442	2487.47	365	332.40	3192	3121.35
Standard Deviation	15.18	100.84	448.44	23.54	234.69	177.78	28.94	32.43	276.75	369.45

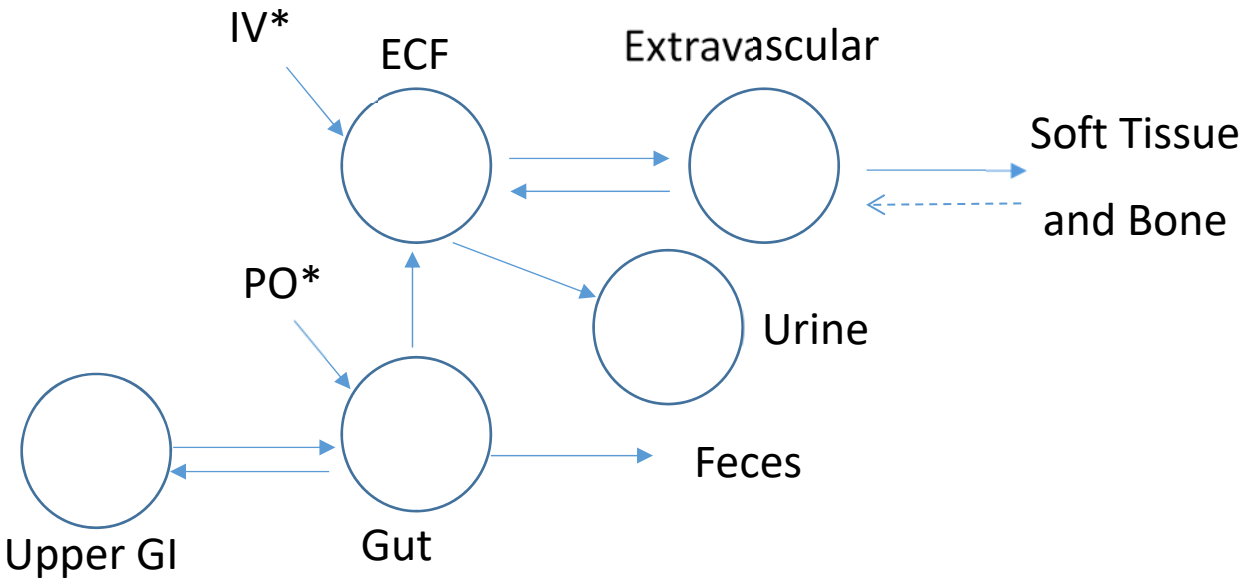


Figure S3.1 Compartmental model of phosphorus metabolism in CKD. This schematic represents the compartmental model developed using the WINSAAM modeling software. This model represents different physiological pools of phosphorus in the body. Absorption was calculated as a ratio of transfer from the 'Gut' to 'ECF' compartment over sum of transfer from the 'Gut' to 'ECF' compartment and into the feces from the 'Gut.' The 'Upper GI' compartment represents a pre-absorptive compartment. IV* indicates IV dose and PO* indicates the oral ^{33}P dose. Solid lines indicate transfer rate was calculated by the model. Dotted lines indicate a physiological pathway that was not calculated due to the length of data sampling. Return of phosphorus from the tissues was represented by entry of phosphorus directly into the 'ECF', shown by the double arrow. The double arrow into the 'Upper GI' represents dietary phosphorus intake.

CHAPTER 4: ADHERENCE TO A CONTROLLED, LOW-PHOSPHORUS DIET MAY DELAY THE POST-DIALYSIS SERUM PHOSPHORUS REBOUND IN HEMODIALYSIS PATIENTS

Abstract

Background Studies have demonstrated poor phosphorus control with thrice weekly hemodialysis (HD). However, these studies did not control for dietary phosphorus intake, nor test blood frequently during the intradialytic interval. We aimed to determine serum phosphorus reduction and rebound over 48-hours in HD patients consuming a controlled, low phosphorus diet.

Methods Serum phosphorus (mg/dL) was analyzed pre-HD and post-HD for 48-hours in patients (N=13) consuming a controlled diet of ~900 mg P/day taking part in a placebo arm of a clinical trial. Subjects had been off phosphate binders for 10 days prior to the study. Linear regression was used to determine relationships between the decline in serum phosphorus post-HD (post-HD drop); 48-hour area under the curve of serum phosphorus (48h AUC); and pre-HD serum phosphorus (Pre-HD). Repeated Measures ANOVA with Dunnett's post-hoc test was used to determine return to pre-HD serum phosphorus levels.

Results Only 2 of 13 subjects returned their pre-HD serum phosphorus within the first 24-hours post-HD, and four of the 13 subjects by 48-hours after the completion of HD. Pre-HD serum phosphorus is positively associated with 48-hr AUC ($P<0.001$). We found an expected correlation ($r= 0.58$; $P= 0.04$) between post-HD drop in serum phosphorus and 48-hour AUC of serum phosphorus post-HD. All serum phosphorus measures in the 48-hours post-HD period were statistically different than pre-HD serum phosphorus ($p<0.0001$) until 48-hours post-HD ($p=0.15$).

Conclusions Adhering to a low phosphorus diet, even in the absence of phosphate binders, may benefit patients receiving HD by delaying post-HD serum phosphorus rebound. Delaying the return of serum phosphorus values to pre-HD levels to between 24-hours and 48-hours post-HD may lessen overall phosphorus exposure and may translate into achieving long-term target serum phosphorus values in HD patients.

Introduction

The dysregulation of phosphorus metabolism in chronic kidney disease (CKD) leads to hyperphosphatemia in end stage renal disease (ESRD) (1-3). Serum phosphorus is regulated by hormones such as parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1,25-dihydroxyvitamin D (1,25D). (4) The reduced ability of the kidney to excrete phosphorus results in chronically high blood levels of FGF23, PTH and low 1,25D. (5, 6) This hormonal profile leaves patients susceptible to an increased risk for fracture, vascular calcification, left ventricular hypertrophy, and higher mortality risk (5, 7-11). Treatments to control serum phosphorus levels in late stage CKD include the prescription of phosphate-binder medications, a low-phosphorus diet, and dialysis (12). However, due to the limited binding capacity of phosphate binders, gastrointestinal side effects, and overall pill-burden associated with CKD and comorbidities, serum phosphorus target levels are often not achieved in dialysis patients (13, 14).

Phosphorus removal during dialysis varies based upon dialysis modality**.** One recent study suggests that hemodialysis may be more effective for phosphorus removal than peritoneal dialysis (15). However, conventional thrice weekly hemodialysis is still not sufficient to reach serum phosphorus goals (16, 17). Other studies have shown more effective management of serum phosphorus using modalities like nocturnal dialysis and short, daily hemodialysis comparative to conventional hemodialysis (18). These more frequent dialysis prescriptions may be more effective due to the rate of phosphorus moving from different physiologic compartments. Studies have shown that there is a rapid decline in serum phosphorus during the initial phases of hemodialysis, but removal plateaus between 2 and 4 hours of dialysis (19). During the short time period of conventional hemodialysis (~4 hours), phosphorus is almost exclusively removed from the blood, and not from other tissue pools of phosphorus (20). Due to the ineffective removal of phosphorus from all physiological compartments during conventional hemodialysis, phosphorus fluxes from the intracellular compartments back into the blood, in the hours post-dialysis, which is termed the serum phosphorus rebound.

Factors affecting the serum phosphorus rebound, and subsequent effects of large shifts in serum phosphorus, on patient health are understudied. Estimation of phosphorus exposure in conventional hemodialysis over time has historically been assessed by the trapezoidal rule using singular blood draws over several months. This is termed time-averaged serum phosphorus (11). In 2012, Viaene and colleagues (21) proposed the use of a time-averaged serum phosphorus, positing that phosphorus toxicity in hemodialysis cannot be determined without considering the daily variation in serum phosphorus in dialysis patients. Previous studies of the serum phosphorus rebound in hemodialysis

patients have shown a return to pre-dialysis serum phosphorus values as soon as 4-24 hours post-dialysis (22, 23). However, these studies did not control dietary phosphorus intake in the hemodialysis patients, nor test blood frequently during the days post-dialysis to determine overall phosphorus reduction and rebound. The aim of this study was to determine serum phosphorus reduction and rebound over a 48-hour period in hemodialysis patients consuming a controlled, low phosphorus diet.

Materials and Methods

Study Design

The current study is a secondary analysis of data from the placebo arm of randomized controlled trial that included a controlled diet. Briefly, the parent study aimed to test safety and the efficacy of a pan-inhibitor of intestinal sodium-dependent phosphate transporters in the reduction of intestinal phosphorus absorption. Hemodialysis patients with Stage 5D CKD were recruited to participate. Enrolled subjects consumed a controlled diet for 10 days and were then admitted into the Indiana Clinical Research Center (CRC) at University Hospital (Indianapolis, IN) for 2 days for a phosphorus absorption testing protocol that included frequent blood draws. All meals were designed to provide approximately 900 mg phosphorus per day. Subjects were admitted as inpatients to the CRC on study day 11 for a 48-hour inpatient stay after completing an early morning ~4 hour dialysis session. Over the 48-hours post-dialysis, serial blood draws were taken for the main study of intestinal phosphorus absorption assessment. The day 11 time points included pre-dialysis, post-dialysis/pre-oral 33P dose, 15 min post-oral dose, 30, 60, 90, 2hr, 2.5, 3, 3.5, 4, 6, and 24 hours post-oral dose on study day 12. Another blood draw was taken immediately prior to receiving the IV 33P dose (pre-IV) and with the same blood sampling schedule as the day prior. Subjects were discharged on study day 13 after providing a final blood draw. Inclusion and exclusion criteria for patients is provided in the Supplemental Material.

The current study utilized data from N=13 subjects from the placebo arm of the parent study investigating intestinal phosphorus absorption in Stage 5D CKD. Pre-dialysis serum phosphorus and the sequential blood draws over the 48-hour inpatient stay were used to study patients' serum phosphorus rebound during the 48-hours post-dialysis. Patients discontinued any phosphate binder medications for 10 days prior to the start of the parent study. This equated to a total of 23 days where N=13 subjects in this study were not taking phosphate binder medication prior to serial blood sampling. Patients were considered to have "returned to baseline" when their post-dialysis serum phosphorus

values were greater than or equal to 100% of the pre-dialysis serum phosphorus value over the 48-hour period.

Other outcome measures included Pre-HD serum phosphorus (Pre-HD in mg/dL), drop in serum phosphorus post-HD (post-HD drop in mg/dL) and 48hr serum phosphorus area under the curve (48hr AUC). Pre-HD serum phosphorus was drawn immediately prior to dialysis session and was analyzed by the Indiana University Clinical Pathology Laboratory. Post-HD serum phosphorus was sampled immediately upon check-in as an inpatient to the CRC, after their morning dialysis session was completed and before any other study activities were completed. These samples were also analyzed at the Indiana University Clinical Pathology Laboratory. Post-HD drop was calculated as:

pre-HD (mg/dL) - post-dialysis serum phosphorus (mg/dL) = post-HD drop (mg/dL)

The trapezoidal rule was used to calculate 48hr AUC. All serum phosphorus values were analyzed by the Indiana University Pathology Laboratories (Indianapolis, IN).

Low Phosphorus Diet

As a part of the parent study, subjects consumed a controlled, low phosphorus diet designed by a registered dietitian bionutritionist for 13 days. All food was prepared in a metabolic kitchen using precise measures of all raw ingredients, 0.1 gram. The study diet was designed to provide approximately 900 mg of phosphorus per day, in accordance with clinical guidelines for dietary phosphorus restriction in ESRD (12). For study days 1-9, subjects consumed their meals as outpatients and were provided with pack-out meals from the Indiana Clinical Research Center (Indianapolis, IN).

Biochemistries

Baseline biochemistries and serum phosphorus during the intradialytic period was analyzed by Indiana University Clinical Pathology Laboratory.

Statistical Analysis

Descriptive statistics were performed for the 13 subjects. Linear regression (PROC GLM) was used to determine relationships among variables. A repeated measures ANOVA was performed with ‘Time’ as a fixed variable, ‘Subject’ as a random variable, and a Dunnett’s post-hoc test (24) to

compare subjects "return to baseline" for serum phosphorus over 48-hours compared to pre-dialysis serum phosphorus. Significance was set at α=0.05. SAS 9.4 (SAS Institute, Cary, NC) was used to perform all statistical analyses.

Results

Patient Demographics and Characteristics

Table 4.1 describes HD patient baseline characteristics of N = 13 subjects in the placebo arm of the parent study. Values reported in this study are from the time of screening. Serum biochemistries shown in table 1 were within the expected range for a CKD 5D patient.

Serum phosphorus Rebound

On average, HD patients had a -2.85 mg/dL drop in serum phosphorus post-dialysis. **Figure 4.1** describes the post-HD drop by study subject, measured within ~1 hour post-dialysis. Only 2 of 13 subjects returned to their pre-HD serum phosphorus within the first 24-hours post-HD. Four of the 13 subjects had returned to their pre-HD serum phosphorus value by 48-hours after the completion of HD. **Table 4.2** describes in detail each patients' 48-hour serum- phosphorus rebound during intradialytic period at 2 hours, 4, 24 and 48-hours.

There is a positive correlation between pre-HD serum phosphorus and 48-hour AUC (r=0.76; P=0.003). Additionally, we found an expected correlation (r= 0.58; P= 0.04) between post-HD drop in serum phosphorus and 48h AUC of serum phosphorus post-HD, seen in **Figure 4.2**.

When compared to the average pre-dialysis serum phosphorus, all serum phosphorus measures (averages for N=13) in the 48h post-HD period were statistically different than pre-HD serum phosphorus (P<0.0001) until 48h post-HD (P=0.15). **Figure 4.3** describes the average serum phosphorus rebound over the 48-hour intradialytic period for all 13 subjects.

Discussion

In this study, we show that in the context of a controlled, low phosphorus diet, even in the absence of phosphate binding medications, serum phosphorus appears to have a delayed rebound post-dialysis. This may translate to a benefit to phosphorus control for HD patients. While our study does not have a high (liberal, or non-controlled) phosphorus diet control comparison, prior

studies on intradialytic serum phosphorus rebound that didn't control dietary phosphorus have shown a return to pre-dialysis values on a much shorter time interval. De Soi and colleagues showed that six, stable HD patients returned to 88-100% of their pre-dialysis serum phosphorus within 4 hours post-dialysis (22). Patient-recorded food diaries reported in this study an average intake of ~800 mg P/day, but the limitations of food diaries in nutrient assessment are well established (25). Therefore, it is likely that this value may have been even higher than patient reports.

Minutolo and colleagues (23) performed a study on post-dialytic rebound of serum phosphorus comparing standard hemodialysis (HD) to hemodiafiltration (HDF) over a three month period. Patients maintained their phosphate binder prescription during the study period. Serial blood draws were also taken, however only statistically compared to their post-dialysis serum phosphorus value, not their pre-dialysis value. Interestingly, graphical representation of their data indicates a possible return to pre-dialysis serum phosphorus at 24-hours for HDF. However, more sampling time-points would be required to discern an exact "return-to-baseline" and an appropriate statistical comparison.

Others have established the general impact of adherence to a low-phosphorus diet and/or limiting phosphorus absorption can have on serum phosphorus and overall phosphate control in HD (26-29). Specifically, studies have shown that patient adherence to a low phosphorus diet can be increased by consistent education from clinicians (30, 31). Another study by Mazzetti and colleagues (32) found that patients who consumed dietary phosphorus within one hour prior to a hemodialysis session experiences alterations in parathyroid hormone levels and had significantly higher serum phosphorus values compared to patients who did not consume phosphorus prior to dialysis (32). These results, combined with our data, illustrate the importance of controlling dietary phosphorus intake to reduce serum phosphorus in HD patients.

Accurate assessment of serum phosphorus in HD a critical factor in determining efficacy of study phosphorus-lowering interventions. However, assessment is challenged due to the alterations in the diurnal pattern of serum phosphorus (21) and variation in serum biomarkers of mineral metabolism attributed to varying stages of CKD-Mineral Bone Disorder (CKD-MBD) (33). Another misleading factor is the assessment of serum phosphorus is that values measured by biochemical assays represent only a miniscule proportion of total body phosphorus pools (34, 35). Frequent blood tests during the intradialytic period, as performed in our study, may be a critical

aspect to gain a more dynamic perspective of phosphorus movement post-dialysis and its correlation with long-term health outcomes in HD.

One limitation of our study include the potential of the post-dialysis rebound beginning prior to the patients' first blood draw in the CRC, or what is considered the "post-dialysis" blood draw for these patients. If this was the case, the post-dialysis drop would be skewed to seem smaller. An additional limitation is that we did not have a true control comparator, or a control group of HD patients consuming a usual- or high-phosphorus diet. However, our study data combine both aspects of dietary adherence (through a controlled study diet) and frequent blood sampling to suggest that consistent adherence to a low-phosphorus diet may aid in the delay of the serum phosphorus rebound during the intradialytic period. This benefit may be conferred even in the absence of phosphate binder medications in HD patients. If maintained, this may translate into reduced patient risk for cardiovascular disease by achieving lower serum phosphorus in HD. Further studies of longer duration and with a control arm are needed during multiple intradialytic periods in HD patients to provide time-averaged concentration serum phosphorus values (11, 36-38) for a better understanding of the potential impact of adherence to a low phosphorus diet on phosphate-lowering in patients undergoing HD.

Table 4.1. Demographics for HD Patients N = 13.

Characteristic	***N***
Female/Male	7/6
Black/White	11/2
Characteristic	*Mean ± SD*
Age, y	51 ± 9
eGFR, mL/min/1.73 m^2	5.2 ± 1.4
Serum Creatinine, mg/dL	10.9 ± 3.2
Serum phosphorus, mg/dL	5.4 ± 1.2

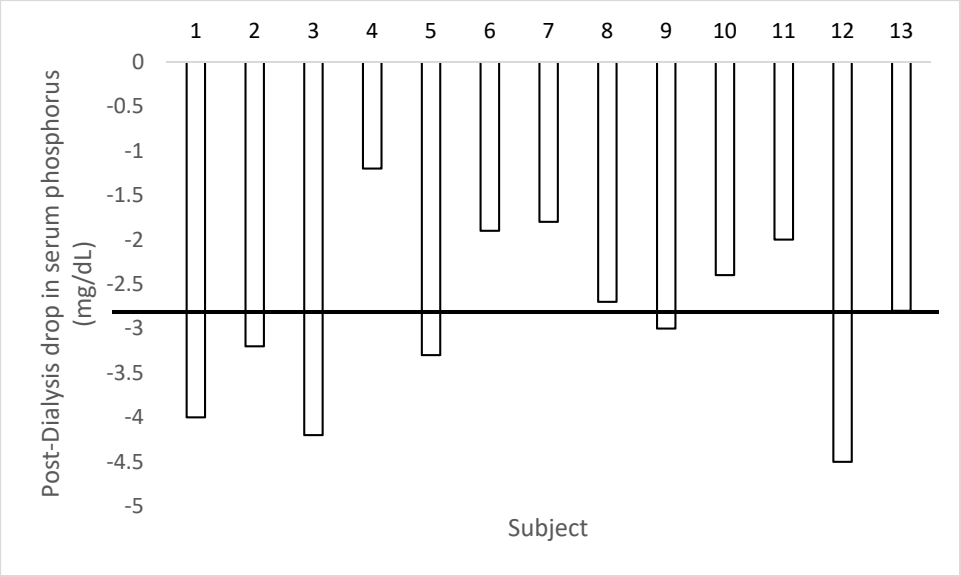


Figure 4.1. Pre-to Post-Dialysis Drop in Serum Phosphorus in mg/dL, by Subject. Black line indicates average drop in serum phosphorus after dialysis, -2.85 mg/dL.

Table 4.2. 48h Serum Phosphorus Rebound During Intradialytic Period. Serum phosphorus (mg/dL) pre-dialysis, post-dialysis, 2h post-HD, 4h post-HD, 24h post-HD, and 48h post-HD. % of pre-HD level is given for the 24h and 48h time point.

Subject	Pre-HD	Post-HD	2hr Post-HD	4hr Post-HD	% rebound of Pre-HD serum phosphorus in 4 hrs	24hr Post HD	% rebound of Pre-HD serum phosphorus in 24 hrs	48hr Post HD	% rebound of Pre-HD serum phosphorus in 48 hrs
1	6.9	2.9	3.6	4.2	61%	5.8	84%	6.5	94%
2	6.4	3.2	3.5	3.9	61%	5.2	81%	5.8	91%
3	9.5	5.3	6	6.3	66%	7.1	75%	8.6	91%
4	4	2.8	1.8	2.1	52%	3.6	90%	4	100%
5	6.1	2.8	2.9	3.5	57%	4.9	80%	5.1	84%
6	5.5	3.6	3.6	4.1	75%	4.4	80%	5.2	95%
7	5.5	3.7	2.8	4	73%	5.5	100%	5.6	102%
8	6.1	3.4	3.4	4.2	69%	5.6	92%	5.7	93%
9	5.7	2.7	2.6	3.2	56%	.	N/A	3.7	65%
10	5.3	2.9	2.7	3.3	62%	4.5	85%	6	113%
11	5.2	3.2	3.8	4.1	78%	5.5	106%	5.5	106%
12	7.9	3.4	4.6	4.8	61%	6.7	85%	7.6	96%
13	6.3	3.5	3.9	4.9	78%	5.7	90%	6.1	97%

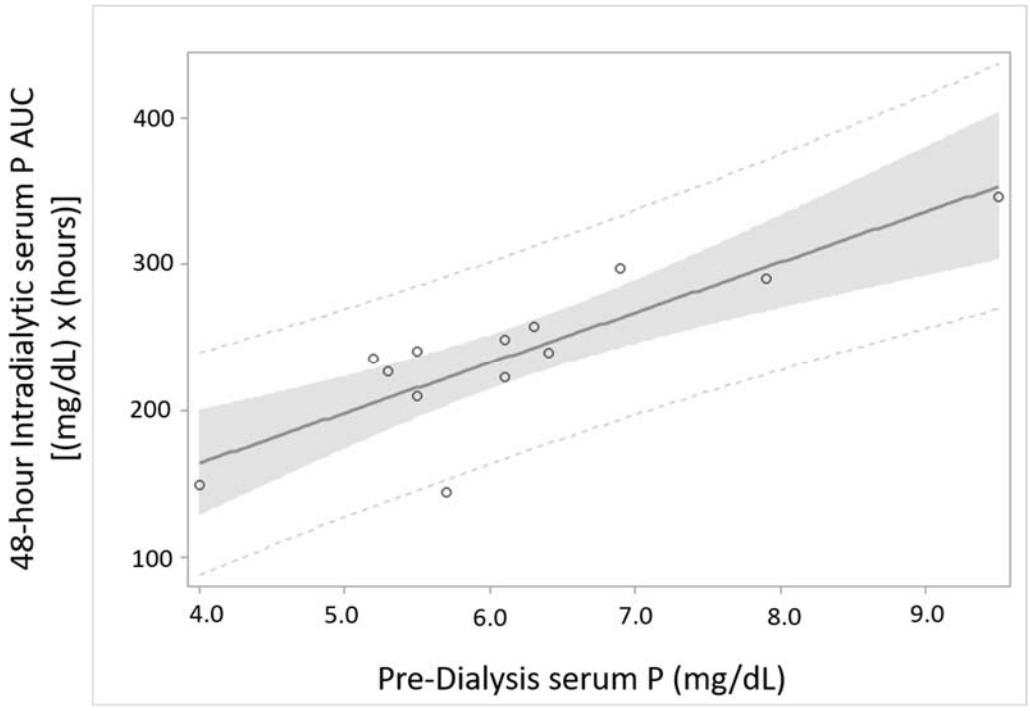


Figure 4.2. Pre-Dialysis Serum Phosphorus is Positively Associated with Overall Serum Phosphorus AUC over 48h Post-dialysis

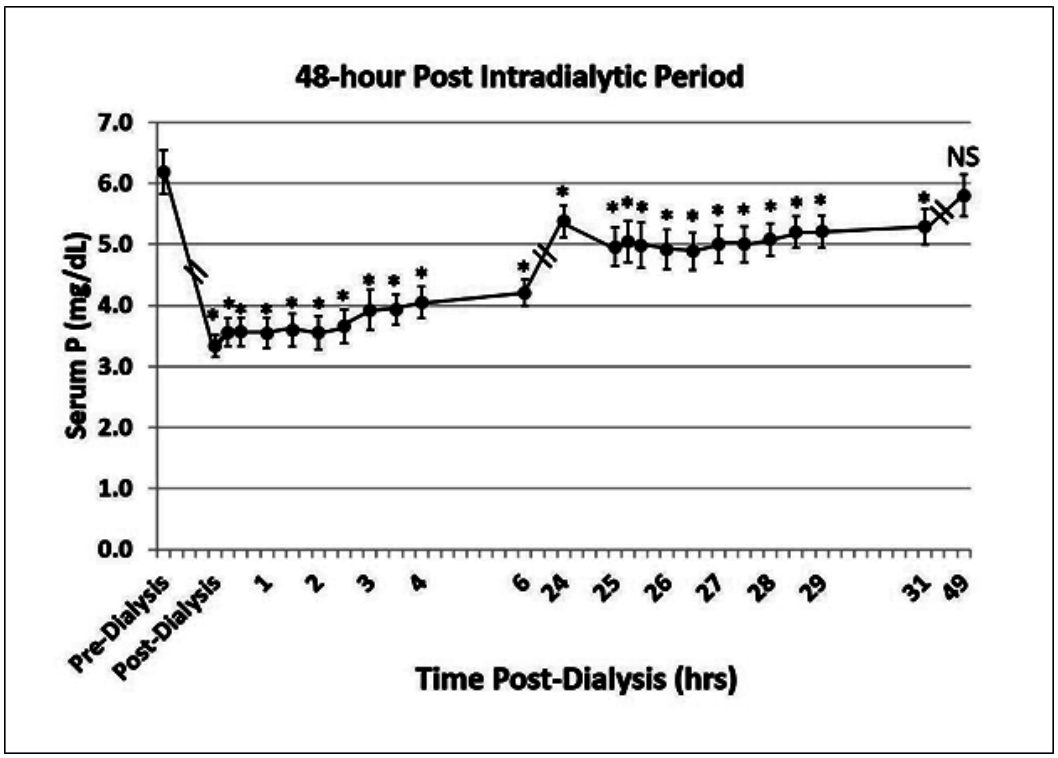


Figure 4.3. 48-Hour Serum phosphorus in HD patients. Mean +/- SEM of serum phosphorus for N=13 ESRD patients at time points through 48h post-dialysis. Mean values were significantly different from mean pre-HD serum phosphorus until 48h post-dialysis.

References

1. Kestenbaum B. Mineral metabolism disorders in chronic kidney disease. JAMA. 2011;305(11):1138-9.
2. Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol. 2004;15(8):2208-18.
3. Palmer SC, Hayen A, Macaskill P, Pellegrini F, Craig JC, Elder GJ, et al. Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. JAMA. 2011;305(11):1119-27.
4. Berndt T, Kumar R. Novel mechanisms in the regulation of phosphorus homeostasis. Physiology (Bethesda). 2009;24:17-25.
5. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. The Journal of clinical investigation. 2011;121(11):4393-408.
6. Wolf M. Forging forward with 10 burning questions on FGF23 in kidney disease. J Am Soc Nephrol. 2010;21(9):1427-35.
7. Chen NX, Moe SM. Pathophysiology of Vascular Calcification. Curr Osteoporos Rep. 2015;13(6):372-80.
8. Rodelo-Haad C, Rodriguez-Ortiz ME, Martin-Malo A, Pendon-Ruiz de Mier MV, Aguera ML, Munoz-Castaneda JR, et al. Phosphate control in reducing FGF23 levels in hemodialysis patients. PLoS One. 2018;13(8):e0201537.
9. Kates DM, Sherrard DJ, Andress DL. Evidence that serum phosphorushosphate is independently associated with serum phosphorusTH in patients with chronic renal failure. Am J Kidney Dis. 1997;30(6):809-13.
10. Floege J, Kim J, Ireland E, Chazot C, Drueke T, de Francisco A, et al. Serum iPTH, calcium and phosphate, and the risk of mortality in a European haemodialysis population. Nephrol Dial Transplant. 2011;26(6):1948-55.
11. Chang WX, Xu N, Kumagai T, Shiraishi T, Kikuyama T, Omizo H, et al. The Impact of Normal Range of Serum phosphorus on the Incidence of End-Stage Renal Disease by A Propensity Score Analysis. PLoS One. 2016;11(4):e0154469.

12. Kidney Disease: Improving Global Outcomes CKDMBDUWG. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney Int Suppl (2011). 2017;7(1):1-59.
13. Waheed AA, Pedraza F, Lenz O, Isakova T. Phosphate control in end-stage renal disease: barriers and opportunities. Nephrol Dial Transplant. 2013;28(12):2961-8.
14. Coladonato JA. Control of hyperphosphatemia among patients with ESRD. J Am Soc Nephrol. 2005;16 Suppl 2:S107-14.
15. Evenepoel P, Meijers BK, Bammens B, Viaene L, Claes K, Sprangers B, et al. Phosphorus metabolism in peritoneal dialysis- and haemodialysis-treated patients. Nephrol Dial Transplant. 2016;31(9):1508-14.
16. Hou SH, Zhao J, Ellman CF, Hu J, Griffin Z, Spiegel DM, et al. Calcium and phosphorus fluxes during hemodialysis with low calcium dialysate. Am J Kidney Dis. 1991;18(2):217-24.
17. Umeukeje EM, Mixon AS, Cavanaugh KL. Phosphate-control adherence in hemodialysis patients: current perspectives. Patient Prefer Adherence. 2018;12:1175-91.
18. Kooienga L. Phosphorus balance with daily dialysis. Semin Dial. 2007;20(4):342-5.
19. Kuhlmann MK. Phosphate elimination in modalities of hemodialysis and peritoneal dialysis. Blood Purif. 2010;29(2):137-44.
20. Daugirdas JT. Removal of Phosphorus by Hemodialysis. Semin Dial. 2015;28(6):620-3.
21. Viaene L, Meijers B, Vanrenterghem Y, Evenepoel P. Daytime rhythm and treatment-related fluctuations of serum phosphorus concentration in dialysis patients. Am J Nephrol. 2012;35(3):242-8.
22. DeSoi CA, Umans JG. Phosphate kinetics during high-flux hemodialysis. J Am Soc Nephrol. 1993;4(5):1214-8.
23. Minutolo R, Bellizzi V, Cioffi M, Iodice C, Giannattasio P, Andreucci M, et al. Postdialytic rebound of serum phosphorus: pathogenetic and clinical insights. J Am Soc Nephrol. 2002;13(4):1046-54.
24. McHugh ML. Multiple comparison analysis testing in ANOVA. Biochem Med (Zagreb). 2011;21(3):203-9.

25. Naska A, Lagiou A, Lagiou P. Dietary assessment methods in epidemiological research: current state of the art and future prospects. F1000Res. 2017;6:926.
26. Block GA, Wheeler DC, Persky MS, Kestenbaum B, Ketteler M, Spiegel DM, et al. Effects of phosphate binders in moderate CKD. J Am Soc Nephrol. 2012;23(8):1407-15.
27. Chertow GM, Burke SK, Raggi P, Treat to Goal Working G. Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. Kidney Int. 2002;62(1):245-52.
28. Gutierrez OM, Wolf M. Dietary phosphorus restriction in advanced chronic kidney disease: merits, challenges, and emerging strategies. Semin Dial. 2010;23(4):401-6.
29. Uribarri J. Phosphorus homeostasis in normal health and in chronic kidney disease patients with special emphasis on dietary phosphorus intake. Semin Dial. 2007;20(4):295-301.
30. Caldeira D, Amaral T, David C, Sampaio C. Educational strategies to reduce serum phosphorus in hyperphosphatemic patients with chronic kidney disease: systematic review with meta-analysis. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2011;21(4):285-94.
31. Tsai WC, Yang JY, Luan CC, Wang YJ, Lai YC, Liu LC, et al. Additional benefit of dietitian involvement in dialysis staffs-led diet education on uncontrolled hyperphosphatemia in hemodialysis patients. Clin Exp Nephrol. 2016;20(5):815-21.
32. Mazzetti T, Hopman WM, Couture L, Christilaw E, Munroe J, Babiolakis CS, et al. Phosphorus Consumption Within 1 Hour Prior to Blood Work and Associated Serum Levels of Phosphate, Calcium, and PTH in Adult Patients Receiving Hemodialysis Treatment. Can J Kidney Health Dis. 2019;6:2054358119856891.
33. Jovanovich A, Kendrick J. Personalized Management of Bone and Mineral Disorders and Precision Medicine in End-Stage Kidney Disease. Semin Nephrol. 2018;38(4):397-409.
34. Medicine Io. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, D.C.: National Academy Press; 1997. 432 p.
35. Moorthi RN, Moe SM. CKD-mineral and bone disorder: core curriculum 2011. Am J Kidney Dis. 2011;58(6):1022-36.

36. Kumar VA, Tilluckdharry N, Xue H, Sidell MA. Serum phosphorus Levels, Race, and Socioeconomic Status in Incident Hemodialysis Patients. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2016;26(1):10-7.
37. Lertdumrongluk P, Rhee CM, Park J, Lau WL, Moradi H, Jing J, et al. Association of serum phosphorus concentration with mortality in elderly and nonelderly hemodialysis patients. Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation. 2013;23(6):411-21.
38. Sim JJ, Bhandari SK, Smith N, Chung J, Liu IL, Jacobsen SJ, et al. Phosphorus and risk of renal failure in subjects with normal renal function. Am J Med. 2013;126(4):311-8.

Supplemental Information

Table S4.1 Subject Inclusion and Exclusion Criteria

Inclusion criteria:

1. Able to provide a written informed consent

2. Age ≥18 years

3. Conventional, and stable, hemodialysis prescription for greater than 3 months before screening.

4. Serum phosphorus >5.5 mg/dL at screening.

5. Receiving stable doses of medications that may affecting serum phosphorus for at least 4 weeks prior to screening and willing to discontinue medications for the duration of the study

6. No major alterations to dietary patterns in the 4 weeks prior to study activities.

7. For patients of reproductive potential: willing to use reliable means of contraception during study period. The pregnancy test must be negative at baseline assessment before randomization.

Exclusion criteria

1. Serum phosphorus ≥7.0 mg/dL at screening

2. Uncontrolled medical conditions including: diabetes, hypertension, chronic constipation and/or diarrhea

3. Hospitalization for cardiac disease in previous 3 months

4. Evidence of acute or chronic hepatitis or known liver cirrhosis; AST and/or ALT >2.5 times the upper limit of normal (ULN)

5. Any significant (in investigator's judgment) history or concomitant presence of gastrointestinal (GI) disorder, including ileus, bowel obstruction, dysphagia, swallowing disorder, severe GI disability or gastro-duodenal ulcer, motility disorder of the intestines, or any history of serious GI surgery or cholecystectomy that may preclude taking study medication or lead to malabsorption

6. Except for CKD, any serious medical condition or abnormality in clinical laboratory tests and ECG at screening that, in the investigator's judgment, precludes the patient's safe participation in and completion of the study

7. Patient with a positive history for human immunodeficiency virus (HIV)

8. History of malignancy in the last 3 years, other than non-melanoma skin cancer

CHAPTER 5: DISCUSSION

Summary and Synthesis

Twenty-Four-Hour Urinary Phosphorus Excretion Variation in Moderate CKD

In this secondary analysis of a phosphorus balance study (1), moderate CKD patients (N=8) consuming a tightly controlled phosphorus diet (1564±52 mg/d) exhibited significant variation in urinary phosphorus excretion. Subjects had a high degree of both intra- (CV=30%) and inter-subject (37%) variation of both urinary phosphorus excretion and urinary creatinine excretion. Additionally, we found that at least two measures of 24-hour urinary phosphorus were necessary to provide a reliable measure. In this study, we had 13 consecutive measures of 24-hour urine phosphorus collected from an inpatient setting. An average of 13 measures provided >90% reliability. Despite the reliability in the average measure, the day-to-day variation indicates that the relationship between dietary phosphorus intake and/or absorption is not applicable to moderate CKD patients consuming a typical American diet. In fact, when using an equation established for healthy individuals (2) to derive "Predicted Dietary phosphorus Intake" and compare to the known, controlled intake in the patients in this study, the equation both over-predicted and under-predicted actual phosphorus intake by up to 79% and 98%, respectively. Lastly, we did not find this variation in CKD to be related to differences in net intestinal phosphorus absorption. However, there was a significant and negative association between 24hr urine phosphorus and whole-body phosphorus retention, suggesting that 24-hour urine phosphorus results should be interpreted with caution and not assumed to be indicative of intestinal phosphorus absorption in all circumstances.

Fractional Intestinal Phosphorus Absorption in Moderate CKD

We conducted a pilot study of intestinal phosphorus absorption using a direct measure of phosphorus absorption with the radioisotopic tracer, ^{33}Phosphorus. We designed our study to mimic the gold-standard, simultaneous administration of two different isotopes (oral and intravenous administration). However, our study protocol involved an oral administration of ^{33}P as orthophosphate in mineral-free water, followed exactly 25 hours by the IV administration of the same isotope. This modified technique is novel in its use in humans to study intestinal fractional phosphorus absorption and also affords greater patient safety due to the lower-energy properties

of ^{33}P compared to the other available phosphorus isotopes (^{32}P) used in past clinical studies. (3, 4)

Our study is also unique as it was a controlled feeding study that included a high dietary phosphorus intake level that is typical of the US diet (5). Our three-day cycle menu was designed to provide approximately 1500 mg of phosphorus per day. Additionally, to account for age, sex, or racial differences in intestinal phosphorus absorption, we matched healthy participants to enrolled moderate CKD patients. Compartmental modeling of fractional phosphorus absorption indicated that there is no difference between moderate CKD patients and healthy individuals. This was despite significantly lower serum 1,25D in moderate CKD patients compared with their healthy matched controls. In fact, CKD patients had an average fractional phosphorus absorption of 0.69 compared to 0.62 in the healthy adults. This is interesting as our lab previously reported that rats with a progressive model of moderate CKD actually had significantly higher phosphorus absorption efficiency compared to healthy control rats (6). These same rats also had lower serum 1,25D values than the healthy controls.

We also revisited the relationship between twenty-four-hour urinary phosphorus excretion and directly measured intestinal phosphorus absorption with our data from these patients. Importantly, we did not find an association between a two-day average measure 24-hour urine phosphorus and fractional phosphorus absorption in either group, or overall. These data support our previous finding that twenty-four-hour urine phosphorus is not a gold-standard biomarker of intestinal phosphorus absorption in CKD, and that care should be taken in interpreting 24-hour urine phosphorus data particularly in cross-sectional studies.

Intradialytic Serum Phosphorus Rebound in Hemodialysis and Overall Phosphorus Exposure

In this study, we found that adherence to a controlled low-phosphorus diet, in the absence of phosphate binder medications, may reduce the overall phosphorus burden in ESRD patients by delaying the intradialytic serum phosphorus rebound. Previous studies have shown a rebound occurring as soon as 4-24 hours post-dialysis (7, 8). In this secondary analysis of a placebo-arm of a controlled feeding study, serial blood draws from N=13 hemodialysis patients (CKD Stage 5D) were analyzed for serum phosphorus rebound over the intradialytic period. Importantly, patients who were enrolled in the parent study discontinued phosphate binder medications 10 days prior to

beginning the study. With frequent blood sampling over 48-hours post-dialysis, we show that only 2 of 13 subjects returned to their pre-dialysis serum phosphorus at 24-hours post-dialysis and 4 of 13 returning at 48-hours post-dialysis. Frequent blood sampling and adherence to a low-phosphorus diet in the absence of phosphate binders are unique features of our study in this understudied area. If maintained over time, a consistent delay in serum phosphorus rebound in hemodialysis patients may equate to better phosphorus control and decreased cardiovascular and mortality risk. However, this conclusion requires further studies of longer duration and with a control comparator group without a controlled low phosphorus diet.

Strengths and Limitations

Each of our studies have strengths and limitations that were discussed in individuals chapters 2-4. For example, a particular strength of all our clinical studies is that prescribed diets for all participants in each of the three studies were designed carefully and made in a metabolic kitchen to ensure accuracy and consistency. Additionally, patients in all studies were admitted as inpatients to the Indiana CTSI Clinical Research Center where their samples were collected at precise and timed intervals. This allowed for greater quality of urinary collections (for patients who were not anuric) for measurement of twenty-four-hour excretion as well as for tighter control around dietary phosphorus intake. Other studies of intestinal phosphorus absorption in humans are few, and did not control for dietary intake leading up to absorption testing. (3, 9-11). Additionally, we were able to show the reliability of the average measure of two 24-hour urine phosphorus values in our first study. Then, use those data to reassess this relationship in N=8 moderate CKD patients and their healthy matched pairs. Finally, our pilot study on phosphorus absorption used a direct measure of intestinal absorption in CKD and suggested inappropriate maintenance of intestinal phosphorus absorption in the context of depressed serum 1,25D in moderate CKD, which corroborated our previous study in rats (6).

The controlled diet and frequent blood testing involved during the inpatient protocol for our study of serum phosphorus rebound in the intradialytic period of hemodialysis patients was a particular strength of that study. Hemodialysis patients struggle to maintain dietary phosphorus restriction while also consuming enough dietary protein. (12, 13) Their participation in the parent study that provided them with all of their meals allowed for a unique opportunity for patients to be

easily compliant to a low-phosphorus diet and determine its effect on the intradialytic serum phosphorus rebound.

However, our studies are also limited in generalizability in some respects. Although dietary phosphorus control is a strength in study design, it also limits applicability to the public as a whole as only one intake level was studied. Not only this, but in the study on twenty-four-hour urine and net phosphorus absorption in moderate CKD, only 8 patients were used in this analysis. While data quality was high in terms of total number of urinary phosphorus measures and precision in collection, the parent study was not powered to determine differences or relationships urinary phosphorus and phosphorus absorption or retention. However, our results from this study complement other studies investigating twenty-four-hour urine phosphorus excretion and overall cardiovascular risk (14) indicating that increased phosphorus excretion in the urine may be related to overall negative phosphorus balance and thus a decreased risk for cardiovascular events resulting from excess phosphorus burden in CKD.

One limitation of our pilot study of intestinal phosphorus absorption in moderate CKD and in healthy adults is the interpretation of a two-day balance period. Spiegel and colleagues (15) showed similar variation in 24-hour urine phosphorus and similar results in their balance study in calcium and phosphorus in CKD as previous data from a full 13-day balance study of Hill et al. in 2013, suggesting that short-term balance studies may yield reliable results. However, important factors such as gastrointestinal transit time and loss of fecal sample during collection can make the interpretation of these data problematic over such a short period of time. For example, in our study, CKD patients had a significantly large number of inpatient stool samples than healthy individuals (CKD= 3.125 v. Healthy=1.5; P=0.0034). Higher number of fecal sample could mean increased fecal phosphorus lost during collection, thus affecting overall phosphorus balance. Not only this, but because of transit time, it is difficult to know what constitutes as 24-hours of fecal phosphorus content when collections took place over just 2 days. These factors may have affected some of our balance calculations and thus must be interpreted with caution.

Lastly, a final limitation regarding our study on serum phosphorus rebound in hemodialysis is the timing of post-dialysis blood draws. Patients did not have a blood draw immediately following their dialysis session but instead had a blood draw immediately upon admission to the clinical research center. Thus, this blood draw was sometimes delayed up to 1 hour after the end of their dialysis session. The duration between completing dialysis and being admitted to the

clinical research center varied based on the patient. Reasons for the delay on patient admission is unclear, but could have been due to patient physical mobility.

Future Directions

Although our research contributes foundational knowledge surrounding intestinal phosphorus absorption in moderate CKD, more work is needed to achieve greater generalizability. Perhaps most importantly is understanding how intestinal phosphorus absorption changes with differing dietary phosphorus intakes in CKD. Particularly, intestinal phosphorus absorption in moderate CKD in the context of a low-phosphorus diet must be understood. We have shown that fractional phosphorus absorption in moderate CKD is similar to healthy individuals despite lower serum 1,25D. Should patients with moderate CKD also have increased absorption in the context of dietary phosphorus restriction, these would be further evidence that a multi-faceted treatment course must be used to decrease phosphorus burden in CKD. Additionally, future work should be done on verifying the potential relationship between 24-hour urine phosphorus and whole-body phosphorus retention. Studies powered to detect differences in phosphorus balance in moderate CKD over a study period of one or two weeks would be valuable in validating this relationship while avoiding the limitations involved with two-day balance studies. Finally, studies measuring time-averaged concentration of serum phosphorus over multiple intradialytic periods while on a low-phosphorus diet are necessary to determine if dietary adherence does indeed decrease overall phosphorus burden in HD.

Conclusions

Intestinal phosphorus absorption in moderate CKD may be inappropriately maintained despite changes in serum 1,25D. Twenty-four-hour urine phosphorus, a previously established biomarker of phosphorus absorption in healthy individuals, is not universally applicable in the moderate-stage CKD patient population where no intervention to affect absorption is taking place. Fractional phosphorus absorption using 33P is similar between moderate CKD patients and healthy controls consuming a controlled diet. This supports our previously reported data from an established rat-model of CKD-MBD (6). Study protocols including controlled feeding and admission to an inpatient study period limit the error introduced by dietary compliance issues and

sample collection. Future studies are needed to determine additional factors influencing phosphorus absorption in moderate CKD in order to drive translational treatments for CKD patients. For ESRD patients, additional studies must be done to determine the impact of lessening phosphorus exposure over-time and its effect on clinical outcomes and mortality.

References

1. Hill KM, Martin BR, Wastney ME, McCabe GP, Moe SM, Weaver CM, et al. Oral calcium carbonate affects calcium but not phosphorus balance in stage 3-4 chronic kidney disease. Kidney Int. 2013;83(5):959-66.
2. Lemann J. Calcium and phosphate metabolism: An overview in health and in calcium stone formers. In: Coe F, Favus M, Pak C, Parks J, Preminger G, editors. Kidney Stones: Medical and Surgical Management. Philadelphia, PA: Lippincott-Raven Publishers; 1996. p. 259-88.
3. Peacock M, Aaron JE, Walker GS, Davison AM. Bone disease and hyperparathyroidism in chronic renal failure: the effect of 1alpha-hydroxyvitamin D3. Clin Endocrinol (Oxf). 1977;7 Suppl:73s-81s.
4. Farrington K, Mohammed MN, Newman SP, Varghese Z, Moorhead JF. Comparison of radioisotope methods for the measurement of phosphate absorption in normal subjects and in patients with chronic renal failure. Clin Sci (Lond). 1981;60(1):55-63.
5. Calvo MS, Sherman RA, Uribarri J. Dietary phosphorushosphate and the Forgotten Kidney Patient: A Critical Need for FDA Regulatory Action. Am J Kidney Dis. 2019;73(4):542-51.
6. Vorland CJ, Biruete A, Lachcik PJ, Srinivasan S, Chen NX, Moe SM, et al. Kidney Disease Progression Does Not Decrease Intestinal Phosphorus Absorption in a Rat Model of Chronic Kidney Disease-Mineral Bone Disorder. J Bone Miner Res. 2019.
7. DeSoi CA, Umans JG. Phosphate kinetics during high-flux hemodialysis. J Am Soc Nephrol. 1993;4(5):1214-8.
8. Minutolo R, Bellizzi V, Cioffi M, Iodice C, Giannattasio P, Andreucci M, et al. Postdialytic rebound of serum phosphorus: pathogenetic and clinical insights. J Am Soc Nephrol. 2002;13(4):1046-54.
9. Davis GR, Zerwekh JE, Parker TF, Krejs GJ, Pak CY, Fordtran JS. Absorption of phosphate in the jejunum of patients with chronic renal failure before and after correction of vitamin D deficiency. Gastroenterology. 1983;85(4):908-16.

10. Condon J. Defective Intestinal Phosphate Absorption in Familial and Non-familial Hypophosphataemia. British Medical Journal. 1970;3:138-41.
11. Scanni R, vonRotz M, Jehle S, Hulter HN, Krapf R. The human response to acute enteral and parenteral phosphate loads. J Am Soc Nephrol. 2014;25(12):2730-9.
12. Bhan I. Phosphate management in chronic kidney disease. Curr Opin Nephrol Hypertens. 2014;23(2):174-9.
13. Milazi M, Bonner A, Douglas C. Effectiveness of educational or behavioral interventions on adherence to phosphate control in adults receiving hemodialysis: a systematic review. JBI Database System Rev Implement Rep. 2017;15(4):971-1010.
14. Palmer SC, Hayen A, Macaskill P, Pellegrini F, Craig JC, Elder GJ, et al. Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. JAMA. 2011;305(11):1119-27.
15. Spiegel DM, Brady K. Calcium balance in normal individuals and in patients with chronic kidney disease on low- and high-calcium diets. Kidney Int. 2012;81(11):1116-22.

APPENDIX A. PROTOCOLS

A1. PROCESSING OF HUMAN URINE SPECIMENS FOR MINERAL ANALYSIS BY ICP

1. For mineral analysis store 1- 8ml tube of acidified urine. Fill up to the line and add 3 drops on concentrated Trace Metal Grade HCL using transfer pipette
2. For ICP analyses, label 15 ml polypropylene conical tubes with subject ID, Date, and Time point. Indicate 11x dilution. Label two tubes for each sample.
3. Add 1 ml of acidified urine and 10 ml of 2% HNO_3. Invert to mix.
4. To make 2% HNO_3: mix 299 ml of 70% Trace Metal Grade HNO_3 with 9700 ml ultra pure water.

A2. PROTOCOL FOR FECAL DIGESTION AND PREPARATION FOR ICP ANALYSIS

Fecal Analysis for P analysis by ICP

1. Using vessels that were previously acid washed (10 ml nitric acid in microwave's cleaning cycle) and dry
2. Plan numbering based on sample number (see layout map)
3. Weigh 0.5 g of fecal sample directly into small crucible
4. Place in oven to dry overnight
5. Acidify sample and wash crucible:
    a. Add 2 mL 70% nitric acid to dissolve dried sample. Allow acid to sit for 30 minutes.
    b. Transfer acidified fecal sample from crucible to microwave vessel using transfer pipette.
    c. Add 3 mL of 70% nitric acid to nearly empty crucible. Pipette acid around crucible to wash and recover remaining fecal sample in crucible. Transfer 3 mL acid to microwave vessel.
    d. Add 2 mL ultrapure to crucible; wash and transfer to microwave vessel.
    e. Add 3 mL ultrapure to crucible; wash and transfer to microwave vessel.
    f. Total volume transferred to vessel should be 10 mL added volume from liquid.
6. Cap tightly (use tool until it clicks) and place into microwave. **If not tight, samples may spurt out the top
7. Use "feces 2" program setting (ramps up to 210 C for 20 minutes, holds for 15 minutes, ramps down for 15 minutes)

For ICP testing

1. Prepare 15 ml centrifuge tubes (Falcon tubes with blue caps) and label them with correct study and sample ID (templates in T drive)
2. Carefully pour samples from vessels into tubes (fizzling is normal). Nitric acid concentration is now ~35%
3. Prepare another set of 15 ml Falcon tubes and label them with correct study and sample ID
4. Transfer 200 ul of the 35% HNO3 solution to the new tubes
5. Fill to 6 ml ultrapure water (HNO3 solution now ~1.1%, total dilution factor is 31x)
6. Close tube with and invert several times to mix

Follow ICP methods to measure P in the axial mode.

Clean Microwave Vessels:

1) Add 10 mL of 70% nitric acid to vessels
2) Select X-Press Clean function.
3) Dispose of acid in appropriate liquid waste container and rinse vessels with ultrapure water

A3. POLYETHYLENE GLYCOL (PEG) TURBIMETRIC ASSAY PROTOCOL

Reagent Supplies	Vendor; Catalog/Sku
Polyethylene Glycol powder (E3350)	OTC Miralax
Gum Arabic Powder	Fisher; ICN15121890
Barium Chloride Anhydrous	Fisher; B31-500
0.3 N Barium Hydroxide	Sigma-Aldrich; B4059-500ML
Zinc Sulfate ($ZnSO4{\cdot}7H_2O$), Granular	Fisher; 02-004-615
80% TCA, Aqueous Solution (will be diluted to 30%)	Fisher; 869316

Lab Equipment:

- P200 pipette and tips
- P1000 pipette and tips
- Serological Pipettes, 15 mL
- Repeater Pipette
  - 2 tips for gum Arabic and TCA reagent
- Volumetric flasks for solutions prep
- 25 mL Erlenmeyer flasks
- Small funnels
- Vortex
- Spectrophotometer

Other Supplies:

- 50 mL falcon tubes
- Disposable glass tubes, 19 mL
  - Fisher; SKU 033415
- Disposable glass tubes, 36 mL
- Polystyrene cuvettes
- Whatman #41 filter paper
  - Fisher; Catalog # 03-341-6

Reagents Prep: Use attached 'Solutions Prep Calculations' template to determine volume of solutions needed based on the number of samples and standard curve. Must do all in duplicates! Prepare all solutions at least 1 day in advance.

1. **Standard PEG solution**. Prepare a 1% (w/v) solution of polyethylene glycol. 1 g of polyethylene dissolved in 100 mL with DDI water. Store in refrigerator. Prepare new solution every two month.
2. **Gum Arabic (12 mg/L).** Store in an amber glass bottle.
3. **BaCl2 (10% w/v).** Weigh 50g of **BaCl2 Anhydrous** and dissolve in 500 mL DDI water

   a. If using **BaCl2 dihydrous**, you must account for the molecular weight of water in the compound. 58.65 g of BaCl2·2H2O dissolved in 500 mL with DDI water.
      i. 5*(10 g*244.26 g/mol /208.26 g/mol) = 58.65 g
      ii. Where '5' is the factor to allow for desired 500 mL volume; 10 g is used because desired w/v is 10%;
      iii. 244.26 g/mol is the molecular weight of BaCl2 dihydrous
      iv. 208.26 g/mol is the molecular weight of BaCl2 minus the 2 waters (244.26-(18 g/mol*2)= 208.26 g/mol)
4. **0.3 N barium hydroxide**. Aqueous solution can be acquired.
   a. If mixing solution from granular barium hydroxide:
      i. 43.301 g of barium hydroxide dissolved to 1 liter with DDI water.
      0.15 M = 0.15 mol/L of barium hydroxide

      0.15 mol *171.34 g/mol = 25.701g

      25.701 g*(171.34g/mol +8*18 g/mol)/171.34=47.301 g

5. **Zinc sulfate ($ZnSO4·7H_2O$), 5% w/v**. 8.90 g $ZnSO4·7H_2O$ Q.S to 100 mL with DDI water.
   a. Equation to account for 7 waters in Zinc Sulfate Heptahydrate:
      i. 5 g*287.38 g/mol /161.38 g/mol=8.90 g
6. **TCA reagent. (30% TCA with 5% BaCl2).** To make 1L TCA reagent: Measure 375 mL of 80% TCA with a graduated cylinder. Pour into a 500 mL beaker. Weigh 50 g of BaCl2 anhydrous and add to 80% TCA. On a heat plate, stir until BaCl2 is dissolved. This will take some time. When BaCl2 is dissolved, transfer to a 1L volumetric flask. Bring solution up to 1L volume. Store solution in amber glass bottle. Allow solution to settle for 1 day until it is clear before using.
   a. **If using granular TCA:** Weigh 300 g TCA in beaker then add some DDI water to dissolve. Transfer to a 1000 mL volumetric flask, add 50 g of BaCl2 (or 58.65 g if using BaCl2 dihydrous) and bring to volume with DDI water. Store in an amber glass bottle. Allow solution to settle for one day so it is clear.

Procedure:

1. Weigh 2.5 g of fecal slurries (duplicates) in clean 50 ml falcon tubes. Dilute up to 50mL with ultrapure water.
2. Make standard PEG solution between 0 and 1.5 mg PEG/mL (duplicates). See table on the next page. Standard curve solutions all go directly into 36ml disposable glass tubes.

PEG standard

Concentration (mg/mL)	1% PEG (µL)	DDI H_2O (µL)
0	-	1000
0.5	50	950
1.0	100	900
2.5	250	750
5.0	500	500
7.5	750	250
10.0	1000	-

Volume of 1% PEG + volume of DDI H_2O=1000 µL=1 mL

3. Dilute standards and blank with 10 mL of DDI water.
4. Prepare samples: Transfer 11 mL of diluted fecal slurry to 36 ml disposable glass tubes. Final volumes for all tubes (blank, standards and samples) in glass tubes should be 11 mL.
5. Add 1 mL $BaCl_2$ and 2 mL barium hydroxide to the blank, standards and diluted samples.
6. Vortex well. Be careful not to allow sample to erupt from the top of the tube.
7. Add 2 mL $ZnSO_4$ and vortex again.
8. Let stand for 10 minutes.
9. For all samples and standard curve: Allow gravity to filter sample into clean 25 mL Erlenmeyer flasks using double thickness (i.e. 2 filter papers) Whatman #41 (12.5 cm diameter) filter paper and plastic funnels. Do not use vacuum filtration because it will pull the barium precipitate through the filter. This takes about 3 hours.
    a. Note that the filtrate in the Erlenmeyer flasks MUST be clear! If it is not, the sample has spilled over the filter into the funnel and has contaminated the filtrate. These samples may not be used.
10. Pipette 1 mL of filtrate (filtrate from fecal samples and standard curve) into 19 ml disposable glass tubes. Using a repeater pipette, add 3 mL gum arabic and 4 mL TCA reagent. Vortex.
11. Let stand for 1-1.5 hours. This allows the emulsion to form.
12. Read the optical density (O.D.) against PEG blank and standards at 650 nm in 1 cm polystyrene cuvettes on a spectrophotometer. The turbidity is not very stable, therefore all readings should be made at the same time after mixing.
13. The OD of the standards is plotted against their PEG concentration. The spectrophotometer will calculate the PEG concentrations of the samples.

A4. SOLUTIONS CALCULATIONS EXAMPLE FOR PEG ASSAY

Solution	**Volume needed/1 sample (mL)**	**Total volume needed per PEG run (mL)**	**Volume to mix and store (mL)**	**Amount needed for correct concentration**	**Actual Weight of reagent**	**Date Mixed**
PEG 1% (for standard curve)*	0.3	25.2	100			
$BaCl_2$	1	84	500			
Barium hydroxide	2	168	1000			
$ZnSO_4$	2	168	500 or 1000			
Gum Arabic**	3	252	500			
TCA reagent#	4	336	1000			
Samples to run for X	42					
Total samples including duplicates	84					
Total samples including standards in duplicate						

APPENDIX B. SAS CODE

Table B1. Sas Code for chapter 3

```
data phosabs;
input ID$ pair$ group$ Pabs mgPabsorbed twentyfourhour_uP calcitriol
twentyfiveD fgf pth avginptmgP avgfecalP twodaybalance numberinptfecal;

datalines;

401   1     CKD   0.35  887   652.3929003 40    33.06891238 44.508297
      95.66666667 1683.278803 1130.510001 -99.62409823      4
601   1     Healthy     0.50  584   826.5156854 28    28.39507531 35.68394037
      58.83333333 1730.785613 174.7165264 729.5534009 1.00
402   2     CKD   0.79  1144  1029.660369 18    29.50854741 72.31732527 90
      1775.523944 124.0077079 621.855867  5.00
502   2     Healthy     0.54  1394  810.9491984 27    26.41871126 51.11777528
      36.83333333 1641.93961  135.4847296 695.505682  3.00
403   3     CKD   0.74  916   347.464524  8     20.65128981 98.57945437
      60.83333333 1233.007236 103.5242575 782.0184549 3.00
603   3     Healthy     0.91  919   963.6186187 27    29.29551513 39.4808747
      33.16666667 1797.448996 254.6155717 579.2148054 2.00
404   4     CKD   0.67  1300  1554.130398 24    46.77256392 113.1695261
      60.83333333 1807.167191 2.455629162 250.5811637 2.00
504   4     Healthy     0.63  1211  960.8384959 46    23.32953749 21.37512305
      43.33333333 1804.488981 192.2479412 651.4025436 1.00
406   5     CKD   0.73  1289  831.5174966 25    30.39351872 90.98558571 132
      1612.322403 160.8635978 619.9413085 3.00
506   5     Healthy     0.52  1178  833.5709942 45    79.45274694 39.5160315
      41.66666667 1774.203207 74.68890924 865.9433033 2.00
407   6     CKD   0.51  752   803.9091874 23    16.49537008 150.2247926
      141.3333333 1530.717849 124.4877176 602.3209437 4.00
507   6     Healthy     0.76  783   786.8063104 44    30.2836094  31.39481086
      42.66666667 1716.469301 85.27910789 844.3838829 1.00
410   7     CKD   0.76  864   942.4238344 34    10.33109176 46.09035297
      172.1666667 1756.259861 177.4358435 636.4001827 3.00
510   7     Healthy     0.72  1327  1229.563639 54    14.09345898 24.46892139
      74    1801.630053 398.0022592 174.0641556 1.00
413   8     CKD   0.96  1644  913.7581809 34    21.67657993 92.70826888
      128.8333333 1771.369542 96.39299003 761.2183715 1.00
513   8     Healthy     0.416 1701  1066.948743 34    35.68512837 25.66425257
      37.5  1807.167191 92.00891084 648.2095376 1.00

;
proc print data=phosabs;
run;
proc glm data=phosabs;
class pair group;
model PAbs = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;

proc sort data= PhosAbs; by group;run;
proc glm; by group;
```

```
model PAbs = twentyfourhour_uP;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;

proc glm data=phosabs;
class pair group;
model twentyfourhour_uP = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;
proc glm data=phosabs;
class pair group;
model calcitriol = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;

proc sort data= PhosAbs; by group;run;
proc glm; by group;
model PAbs = calcitriol;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;
proc glm data=phosabs;
class pair group;
model fgf = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;
proc glm data=phosabs;
class pair group;
model pth = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;

proc print data=phosabs;
run;
proc sort data= PhosAbs; by group;run;
proc glm data= phosabs;by group;
model twodaybalance = twentyfourhour_uP;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;
proc print data=phosabs;
run;
proc glm data=phosabs;
class pair group;
model twodaybalance = pair group;
lsmeans group/pdiff stderr lines;
output out= stdves p=predict r=resid;
run;
```

DEMOGRAPHICS

```
data demographics;
input ID$ pair$ group$ age BMI BUN eGFR serumP serumCa ;

datalines;
401   1     CKD   68    33.92695416 29    42    4.2   9.8
601   1     Healthy     58    36.04950322 14    113   3.3   9.7
402   2     CKD   49    41.08885074 33    37    .     9.4
502   2     Healthy     45    36.67621025 11    85    3.2   9.2
403   3     CKD   66    23.41679522 17    34    3     9.4
603   3     Healthy     59    25.35254679 17    71    3.5   9.5
404   4     CKD   47    .     21    49    2.5   8.8
504   4     Healthy     41    25.71959715 10    90    3.1   9.8
406   5     CKD   67    26.86125437 31    42    3.6   9.3
506   5     Healthy     59    28.15166656 16    90    4.1   9.5
407   6     CKD   69    45.56137136 61    29    3.9   9
507   6     Healthy     69    26.12623822 .     .     .     .
410   7     CKD   56    31.54061289 25    55    2.9   9.3
510   7     Healthy     58    26.26038781 16    78    2.8   10
413   8     CKD   30    19.47111721 38    41    3.4   9.6
513   8     Healthy     30    23.53993623 22    90    3.8   10
;
proc print data= demographics;
run;
proc ttest data=demographics sides=2 alpha=0.05 h0=0;
class group;
var age BMI BUN eGFR serumP serumCa;
run;
```

Table B2. SAS CODE FOR CHAPTER 4

```
data serumP;
input

Subj  AUC_total   postdial_drop     UF    Dialysate_Ca      Predialy_P
      PostDial_P  B_one B_two B_three     B_four      B_five      B_six
      B_seven     B_eight     B_nine      B_ten Oneday_PD   B_twelve
      B_thirt     B_fourt     B_fift      B_sixt      B_sevent    B_eight
      B_nint      B_twenty    B_twone     B_twtwo     Twoday_PD;

datalines;


1001  297.4625    4     2.737 2.5   6.9   2.9   .     .     3.3   3.8   3.6
      3.5   3.8   4     4.2   4.5   5.8   5.7   5.3   5.2   4.9   5     5.3
      5.1   5.2   4.8   5.2   5.2   6.5
1003  239.975     3.2   3.319 2.5   6.4   3.2   3.6   3.6   3.8   3.7   3.5
      3.6   3.6   3.9   3.9   4.2   5.2   5     4.9   5     4.8   5.2   5
      5     5     5.1   5.3   5.5   5.8
1004  346.1875    4.2   2.4   2.5   9.5   5.3   5.8   5.9   6     6.1   6
      6     6.1   6.1   6.3   6.1   7.1   7.4   7.4   7.5   6.9   6.8   6.7
      6.9   7     7.1   7.4   7.8   8.6
```

```
1005  148.4 1.2   3.229 2.5   4     2.8   2.6   2.3   2     1.8   1.8   1.8
      2.1   2.2   2.1   2.4   3.6   2.9   2.6   2.6   2.5   2.6   2.7   2.9
      3.2   3.2   3.3   3.1   4
1006  222.375     3.3   1.5   2.5   6.1   2.8   2.8   3     2.6   2.8   2.9
      3     3.2   3.3   3.5   3.9   4.9   4.6   4.7   4.6   4.7   4.5   4.6
      4.7   4.9   5     5     5.4   5.1
1007  209.5125    1.9   0.5   2     5.5   3.6   3.8   3.7   3.5   3.4   3.6
      3.8   3.9   4     4.1   3.9   4.4   3.8   3.7   3.8   4     4     4.3
      4.2   4.3   4.5   4.5   4.4   5.2
1008  240.675     1.8   4.11  2.5   5.5   3.7   3.9   3.8   3.8   3.8   3.8
      4.1   4.1   3.9   4     4.1   5.5   5.4   5.5   5.3   5     5     4.9
      4.7   4.9   5     5.1   5.5   5.6
1010  248.4 2.7   2.5   2.5   6.1   3.4   3.6   3.5   3.6   3.6   3.4   3.6
      3.9   4.2   4.2   4.4   5.6   5.5   5.7   .     5.2   4.8   5.7   5.6
      5.7   5.7   5.7   5.6   5.7
1011  144.1375    3     4.01  2.5   5.7   2.7   2.9   2.9   2.8   2.7   2.6
      2.7   2.8   3     3.2   3.8   .     3.9   3.8   3.6   3.5   3.5   3.5
      3.6   3.8   .     3.8   4     3.7
1012  226.2875    2.4   3.81  2.5   5.3   2.9   3.1   3.2   3.2   3     2.7
      2.8   3.1   3.3   3.3   3.9   4.5   4.4   4.4   4.6   4.7   4.9   5.1
      5.2   5.1   5.3   5.4   5.2   6
1014  235.3 2     1.6   2.5   5.2   3.2   3.5   3.6   3.6   3.8   3.8   3.8
      3.8   4     4.1   4     5.5   5.3   5.3   5.3   5.3   5.3   5.1   5.1
      5.1   5.2   5.3   5.1   5.5
1021  290.2375    4.5   2.75  2.25  7.9   3.4   3.7   3.9   4.2   4.5   4.6
      4.9   6.6   5     4.8   4.9   6.7   .     6.8   6.8   7.2   6.8   7.2
      6.7   6.5   6.2   6.1   6.1   7.6
1022  257.325     2.8   1.18  2.5   6.3   3.5   3.4   3.4   3.7   3.7   3.9
      3.9   4     4.2   4.9   4.6   5.7   5.6   5.5   5.5   5.2   5.1   5
      5.3   5.3   5.3   5.6   5.8   6.1

;
ods graphics on;
proc glm;
class subj;
model AUC_total = postdial_drop / p clm;
run;

proc glm;
class subj;
model postdial_drop= UF / p clm;
run;

proc glm;
class subj;
model AUC_total= UF / p clm;
run;

proc glm;
class subj;
model AUC_total= Predialy_P / p clm;
run;

proc glm;
class subj;
model UF= Predialy_P / p clm;
run;
```

```
proc glm;
class subj;
model UF= Dialysate_Ca / p clm;
run;
proc glm;
class subj;
model AUC_total= Dialysate_Ca / p clm;
run;

proc means data=serumP;
run;
proc print data=serumP;
run;
data New;
input Subj   T0    T1    T2    T3    T4    T5    T6    T7    T8    T9    T10
      T11   T12   T13   T14   T15   T16   T17   T18   T19   T20   T21   T22
      T23   T24;
datalines;

1001  6.9   2.9   .     .     3.3   3.8   3.6   3.5   3.8   4     4.2   4.5
      5.8   5.7   5.3   5.2   4.9   5     5.3   5.1   5.2   4.8   5.2   5.2
      6.5
1003  6.4   3.2   3.6   3.6   3.8   3.7   3.5   3.6   3.6   3.9   3.9   4.2
      5.2   5     4.9   5     4.8   5.2   5     5     5     5.1   5.3   5.5
      5.8
1004  9.5   5.3   5.8   5.9   6     6.1   6     6     6.1   6.1   6.3   6.1
      7.1   7.4   7.4   7.5   6.9   6.8   6.7   6.9   7     7.1   7.4   7.8
      8.6
1005  4     2.8   2.6   2.3   2     1.8   1.8   1.8   2.1   2.2   2.1   2.4
      3.6   2.9   2.6   2.6   2.5   2.6   2.7   2.9   3.2   3.2   3.3   3.1
      4
1006  6.1   2.8   2.8   3     2.6   2.8   2.9   3     3.2   3.3   3.5   3.9
      4.9   4.6   4.7   4.6   4.7   4.5   4.6   4.7   4.9   5     5     5.4
      5.1
1007  5.5   3.6   3.8   3.7   3.5   3.4   3.6   3.8   3.9   4     4.1   3.9
      4.4   3.8   3.7   3.8   4     4     4.3   4.2   4.3   4.5   4.5   4.4
      5.2
1008  5.5   3.7   3.9   3.8   3.8   3.8   3.8   4.1   4.1   3.9   4     4.1
      5.5   5.4   5.5   5.3   5     5     4.9   4.7   4.9   5     5.1   5.5
      5.6
1010  6.1   3.4   3.6   3.5   3.6   3.6   3.4   3.6   3.9   4.2   4.2   4.4
      5.6   5.5   5.7   .     5.2   4.8   5.7   5.6   5.7   5.7   5.7   5.6
      5.7
1011  5.7   2.7   2.9   2.9   2.8   2.7   2.6   2.7   2.8   3     3.2   3.8
      .     3.9   3.8   3.6   3.5   3.5   3.5   3.6   3.8   .     3.8   4
      3.7
1012  5.3   2.9   3.1   3.2   3.2   3     2.7   2.8   3.1   3.3   3.3   3.9
      4.5   4.4   4.4   4.6   4.7   4.9   5.1   5.2   5.1   5.3   5.4   5.2
      6
1014  5.2   3.2   3.5   3.6   3.6   3.8   3.8   3.8   3.8   4     4.1   4
      5.5   5.3   5.3   5.3   5.3   5.3   5.1   5.1   5.1   5.2   5.3   5.1
      5.5
1021  7.9   3.4   3.7   3.9   4.2   4.5   4.6   4.9   6.6   5     4.8   4.9
      6.7   .     6.8   6.8   7.2   6.8   7.2   6.7   6.5   6.2   6.1   6.1
      7.6
```

```
1022  6.3   3.5   3.4   3.4   3.7   3.7   3.9   3.9   4     4.2   4.9   4.6
      5.7   5.6   5.5   5.5   5.2   5.1   5     5.3   5.3   5.3   5.6   5.8
      6.1
;
proc glm data=New;
class subj;
model T24-T0= Subj / nouni;
repeated Time;
run;
proc sort data=TimedP;
by Subj;
run;
data New(keep=t24-t0 Subj);
array yy(3) T24-t0;
do Time = 0 to 24;
set TimedP;
by Subj;
yy(Time)= sP;
if last.subj then return;
end;
run;
/**correlations from original data set**/
proc corr data=serumP spearman;
var AUC_total     postdial_drop Predialy_P PostDial_P;
run;
ods graphics off;
```

APPENDIX C. POSTER PRESENTATIONS

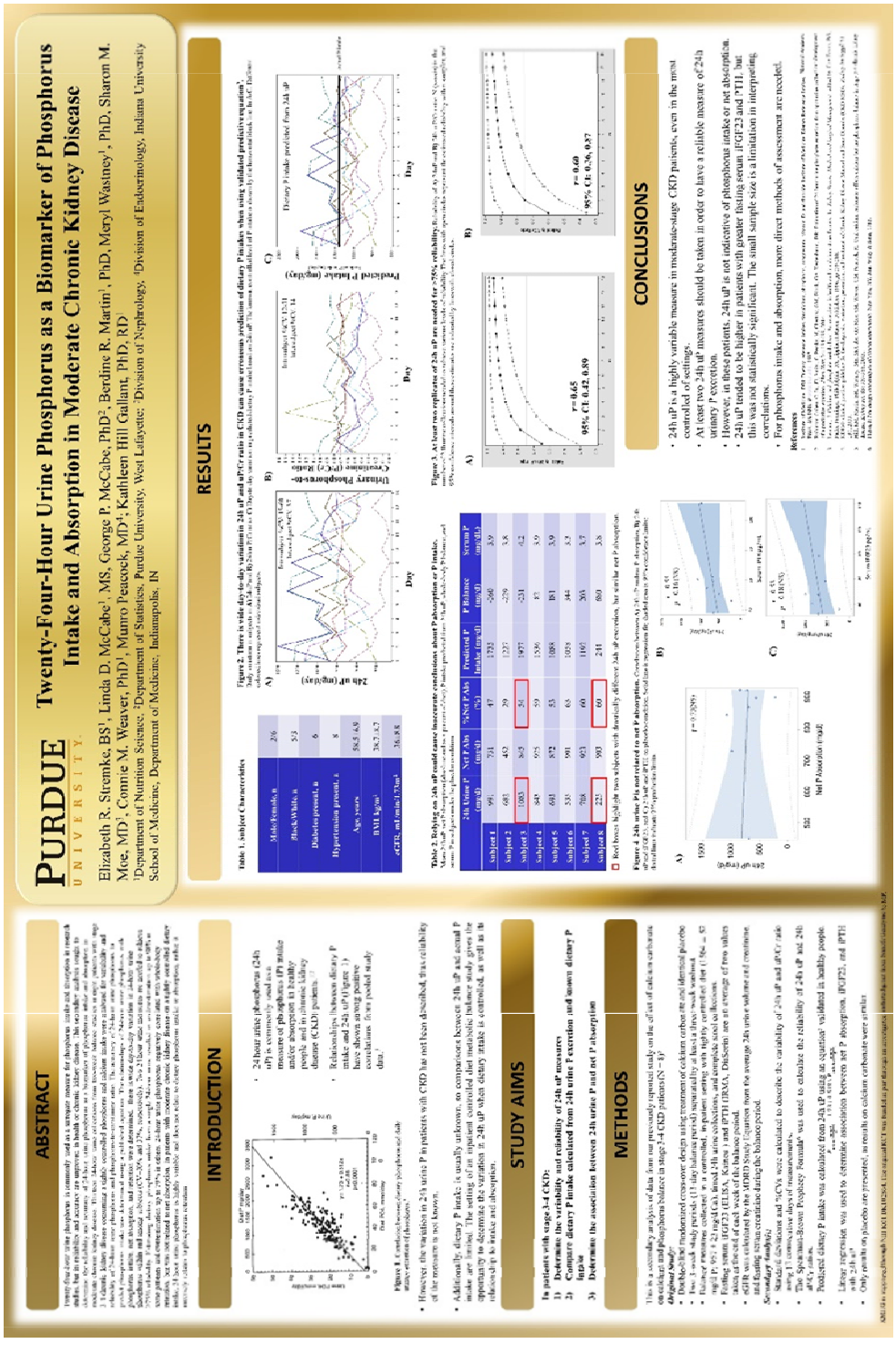


Figure C1. "Twenty-Four-Hour Urine phosphorus as a Biomarker of Phosphorus Intake and Absorption in Moderate Chronic Kidney Disease"

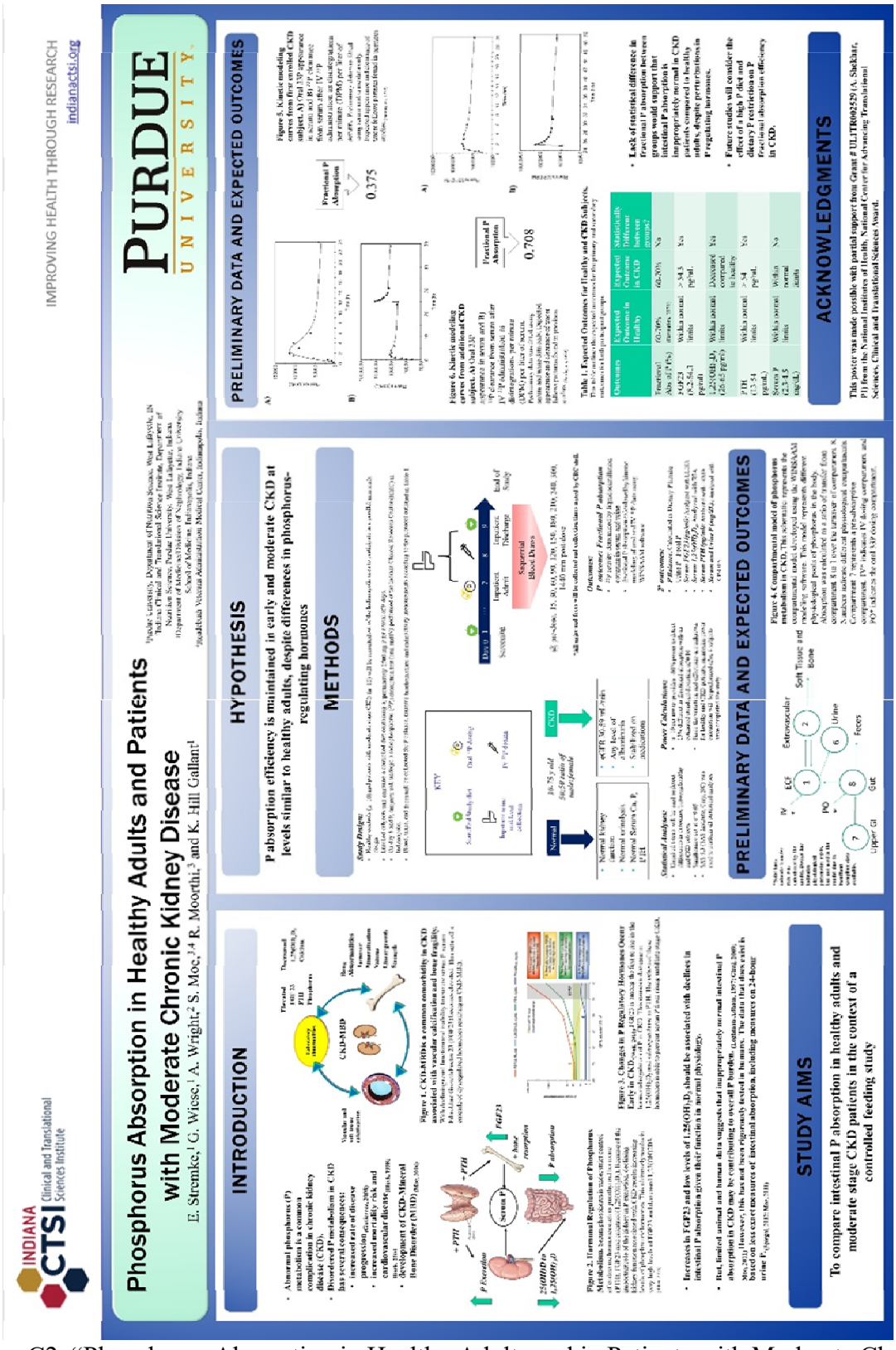


Figure C2. "Phosphorus Absorption in Healthy Adults and in Patients with Moderate Chronic Kidney Disease."

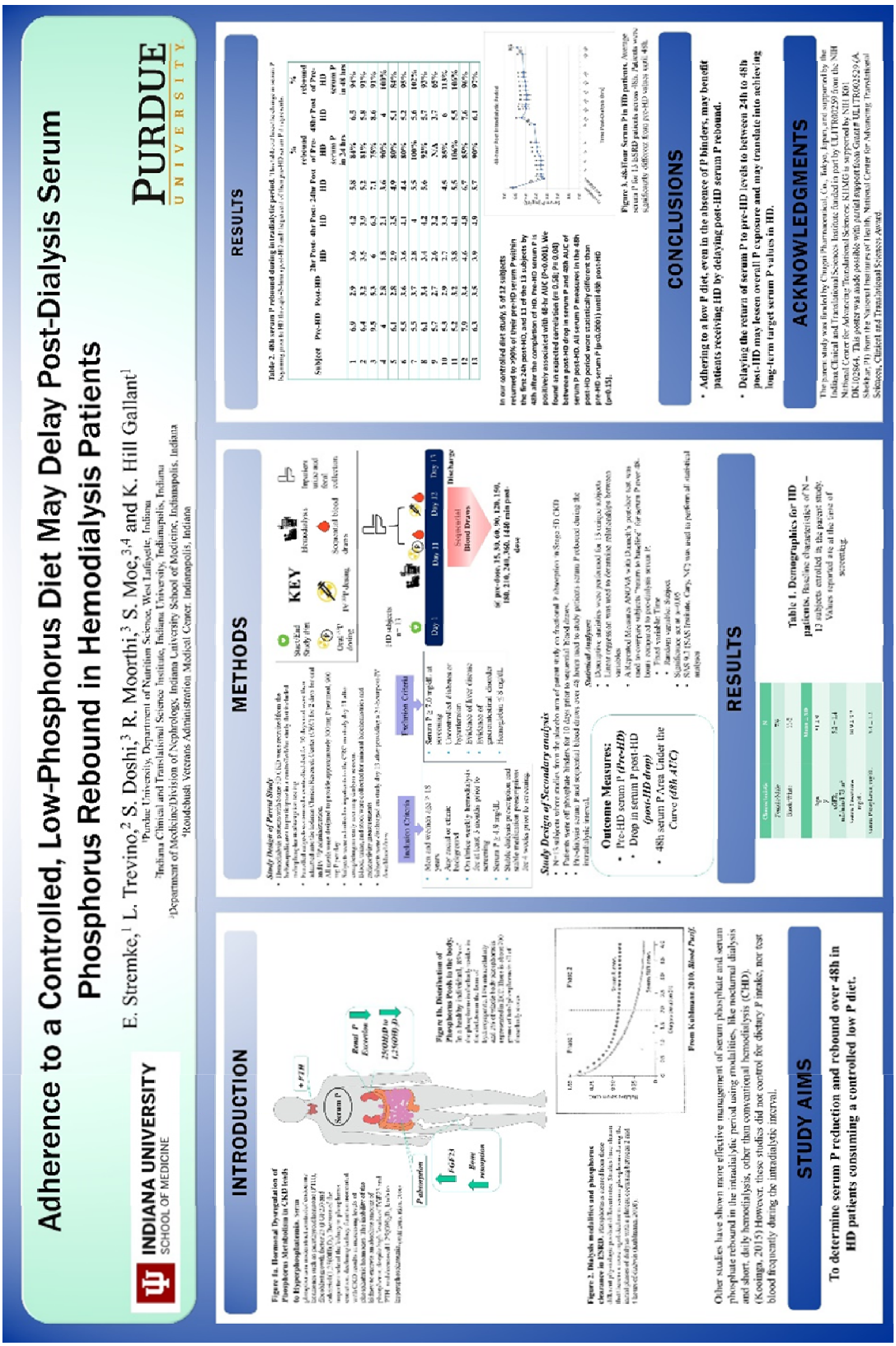


Figure C3. "Adherence to a Controlled Low-phosphorus Diet May Delay Post-dialysis Serum phosphorus Rebound in Hemodialysis Patients."

VITA

Elizabeth R. Stremke, PhD Candidate

EDUCATION

2015-2020 (anticipated) **Doctor of Philosophy**, Nutrition Science
Interdepartmental Nutrition Program (INP)
Purdue University, West Lafayette, IN, USA
Advisor: Kathleen M. Hill Gallant, PhD, RD
Dissertation: Intestinal Phosphorus Absorption in Healthy Individuals and in Patients with Moderate Chronic Kidney Disease

2019-2020 (anticipated) Coordinated Program in Dietetics (CPD)
Purdue University, West Lafayette, IN, USA
Registered Dietitian (RD) exam eligible upon completion

2013-2015 **Bachelor of Science**, Dietetics
Purdue University, West Lafayette, IN, USA

2005-2010 **Bachelor of Science**, Business Management
Purdue University, West Lafayette, IN, USA

RESEARCH EXPERIENCE

2015-present Graduate Research Assistant, Department of Nutrition Science, Purdue University

2013-2015 Undergraduate Research Assistant, Department of Nutrition Science, Purdue University
Hill Gallant Lab

Summer 2013 Undergraduate Research Assistant, Department of Nutrition Science, Purdue University
Mattes Lab

PEER-REVIEWED PUBLICATIONS

1. **Stremke ER**, Hill Gallant KM.
Intestinal Phosphorus Absorption in Chronic Kidney Disease. *Nutrients*. 2018 Sept 23;10(10) doi: 10.3390/nu10101364; PMID: 30249044

2. Choi MS, Kistler B, Wiese GN, **Stremke ER**, Wright AJ, Moe SM, Hill Gallant KM.
Pilot Study of the Effects of High-Protein Meals During Hemodialysis on Intradialytic Hypotension in Patients Undergoing Maintenance Hemodialysis. *J Ren Nutr*. 2018 Aug 11. doi: 10.1053/j.jrn.2018.06.002; PMID: 30107974

3. **Stremke ER**, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, Hill Gallant KM.

Twenty-Four-Hour Urine Phosphorus as a Biomarker of Dietary Phosphorus Intake and Absorption in CKD: A Secondary Analysis from a Controlled Diet Study. *Clin J Am Soc Nephrol*. 2018 July 6;13(7):1002-1012
doi: 10.2215/CJN.00390118; PMID: 29921736

4. Vorland CJ, **Stremke ER**, Moorthi RN, Hill Gallant KM.
Effects of Excessive Dietary Phosphorus Intake on Bone Health. *Curr Osteoporos Rep*. 2017 Oct; 15(5): 473-482
doi: 10.1007/s11914-017-0398-4; PMID: 28840444

MANUSCRIPTS in PROGRESS

5. **Stremke ER**, Biruete A, Hill Gallant KM.
Dietary Protein Intake and Bone Across Stages of Chronic Kidney Disease
Submitted, Curr Osteoporos Rep

6. Hill Gallant KM, **Stremke ER**, Trevino L, Wastney ME, Moe SM.
A Single-Center, Randomized 2-Period Crossover Study to Explore the Safety Pharmacokinetics, Pharmacodynamics and Efficacy of a novel NaPiIIb inhibitor in Patients with Chronic Kidney Disease and Hyperphosphatemia on Hemodialysis.
Submitted, CJASN

7. **Stremke ER**, Wiese GN, Moe SM, Wastney ME, Moorthi RN, Hill Gallant KM.
Fractional Phosphorus Absorption is Inappropriately Normal and Does Not Correlate with 24-Hour Urine Phosphorus in Moderate Chronic Kidney Disease Compared with Healthy Adults.
In Preparation

8. **Stremke ER**, Trevino L, Doshi S, Moorthi R, Moe S, Hill Gallant KM.
48-hour Post-dialysis Serum Phosphate Rebound in Hemodialysis Patients Consuming a Controlled, Low-phosphorus Diet.
In Preparation

9. Lobene AJ, **Stremke ER**, Moe SM, Hill Gallant KM.
Validating Equations for Estimating 24-Hour Urinary Sodium Excretion in Healthy and CKD Patients Using a Controlled Feeding Study
In Preparation

10. Wiese GN, Biruete A, **Stremke ER**, Wright AJ, Moe SM, Moorthi RN, Hill Gallant KM.
Gut-Derived Uremic Retention Solutes in Patients with Chronic Kidney Disease and Healthy Adults.
In Preparation

ABSTRACTS AND PRESENTATIONS

Oral Presentations

1. **Stremke ER**, Wiese GN, Wastney ME, Moe SM, Moorthi RN, Hill Gallant KM. Fractional phosphorus absorption is inappropriately normal and does not correlate with 24 hour urine phosphorus in patients with moderate chronic kidney disease compared with healthy adults. American Society of Nephrology Kidney Week. Washington DC. November 2019. Oral Presentation.

2. **Stremke ER**, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, Hill Gallant KM. Twenty-four-hour urine phosphorus is highly variable in patients with moderate chronic kidney disease on a controlled phosphorus diet. American Society for Nutrition Experimental Biology. Chicago, Illinois. April 2017. Oral Presentation.

Poster Presentations

1. **Stremke ER**, Trevino L, Doshi S, Moorthi R, Moe S, Hill Gallant KM. 48-hour Post-dialysis Serum Phosphate Rebound in Hemodialysis Patients Consuming a Controlled, Low-phosphorus Diet. American Society of Nephrology Kidney Week. Washington, DC. November 2019. Poster Presentation.
2. Wiese GN, Biruete A, **Stremke ER**, Wright AJ, Moe SM, Moorthi RN, Hill Gallant KM. Gut-Derived Uremic Retention Solutes in Patients with Chronic Kidney Disease and Healthy Adults. American Society of Nephrology Kidney Week. Washington, DC. November 2019. Poster Presentation.
3. **Stremke ER**, Wiese GN, Wastney ME, Moe SM, Moorthi RN, Hill Gallant KM. Phosphorus Absorption in Healthy Adults and in Patients with Moderate Chronic Kidney Disease. Association for Clinical and Translational Science Translational Science Meeting. Washington, DC. March 2019. Poster Presentation.
4. **Stremke ER**, Wiese GN, Wastney ME, Moe SM, Moorthi RN, Hill Gallant KM. Phosphorus Absorption in Healthy Adults and in Patients with Moderate Chronic Kidney Disease. Purdue University Health and Disease: Science, Technology, Culture, and Policy. February 2019. Poster Presentation.
5. Hill Gallant KM, **Stremke ER**, Trevino L, Moe SM, Wastney ME. Intestinal Phosphorus Absorption Assessment of Kinetic Modeling of ^{33}P Radiotracer in Hemodialysis Patients. American Society of Nephrology Kidney Week. San Diego, CA. October 2018. Poster Presentation.
6. Hill Gallant KM, Choi MS, **Stremke ER**, McCabe GP, Peacock M, Wastney ME. Evaluation of a Radiophosphorus Method for Intestinal Phosphorus Absorption Assessment in Humans. American Society for Bone and Mineral Research Annual Meeting. Montreal, QC, CA. September 2018.
7. **Stremke ER**, Wiese GN, Wastney ME, Moe SM, Moorthi RN, Hill Gallant KM. Phosphorus Absorption in Healthy Adults and in Patients with Moderate Chronic Kidney Disease. Indiana CTSI Translational Annual Meeting. Indianapolis, Indiana. September 2018. Poster Presentation.
8. **Stremke ER**, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, Hill Gallant KM. Twenty-four-hour urine phosphorus is highly variable in patients with moderate chronic kidney disease on a controlled phosphorus diet. American Society for Nutrition Experimental Biology. Chicago, Illinois. April 2017. Emerging Leaders Poster Presentation.
9. **Stremke ER**, Wiese GN, Wastney ME, Moe SM, Moorthi RN, Hill Gallant KM. Phosphorus Absorption in Healthy Adults and in Patients with Moderate Chronic Kidney Disease. Purdue University Interdepartmental Nutrition Program (INP). West Lafayette, IN. February 2017. Poster Session
10. **Stremke ER**, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, Hill Gallant KM. Twenty-four-hour urine phosphorus is reflective of phosphorus retention, not dietary intake or absorption, in patients with moderate chronic kidney disease. Purdue University Nutrition Science May Conference. West Lafayette, IN. May 2016. Poster Presentation.
11. **Stremke ER**, McCabe LD, McCabe GP, Martin BR, Moe SM, Weaver CM, Peacock M, Hill Gallant KM. Twenty-four-hour urine phosphorus is reflective of phosphorus retention, not dietary intake or

11. absorption, in patients with moderate chronic kidney disease. Purdue University Interdepartmental Nutrition Program. West Lafayette, IN. February 2016. Poster Presentation.
12. **Stremke ER.** Hill Gallant KM. Got Too Much Phosphorus? Why Phosphorus in Your Food Matters. Purdue University Next Generations Scholars. West Lafayette, IN. November 2015. Poster Session.
13. **Stremke ER,** Vorland CJ, Hill Gallant KM. *In-Vitro* Bioaccessibility of Phosphorus in a Mixed Diet. Purdue University Undergraduate Research Colloquium. West Lafayette, IN. April 2015. Poster Session.

Guest Lectures and Seminars

14. **Stremke ER,** Hill Gallant KM. Intestinal Phosphorus Absorption in Moderate Chronic Kidney Disease. Purdue University Interdepartmental Nutrition Program (INP) Seminar Series. West Lafayette, IN. April 2019. Seminar.

HONORS and AWARDS

Health and Disease: Science, Technology, Culture and Policy Poster Competition, Prevention and Wellness/Obesity and Related Disease category, 2nd place, Purdue University

February 2019

Kroenke Travel Award 2018, Indiana Clinical and Translational Science Institute (CTSI) Annual Meeting TL1 Fellows Poster Competition

September 2018

Indiana CTSI TL1 Pre-Doctoral Training Fellowship in Translational Research

July 2018-May 2020

American Society of Nutrition Emerging Leader Award 2017, Vitamins and Minerals Section

April 2017

Travel Award, INP Clinical Poster Competition, Purdue University — February 2016

Ross Doctoral Fellowship, Purdue University — August 2015 – August 2016

Dean's List, College of Health and Human Sciences (HHS), Purdue University — May 2015

Semester Honors, College of HHS, Purdue University — Fall 2013- Spring 2015

HHS Success Mentors Program, Purdue University, mentor — August 2014 – December 2015

Helen B. Schleman Scholarship, Purdue University — August 2014

Nontraditional Student Scholarship, Office of the Dean of Students, Purdue University — August 2014

GRANT WRITING EXPERIENCE

Mock NIH Study Section Participant, Translational Science 2019, Association for Clinical and Translational Science Meeting, facilitated through Indiana CTSI

Pre-Doctoral Fellowship in Translational Research (TL1), Indiana CTSI. *"Phosphorus Absorption in Healthy Adults and in Patients with Chronic Kidney Disease."* 2017, **awarded**, July 2017- May 2020

Pre-Doctoral Fellowship, American Society of Nutrition, "Effects of Low and High Dietary Phosphorus on Phosphorus Absorption in Healthy Adults and in Patients with Early and Moderate Chronic Kidney Disease." 2015, not awarded

TEACHING EXPERIENCE

Purdue University Department of Nutrition Science, West Lafayette, IN

Fall 2016 Graduate Teaching Assistant, NUTR 480 Medical Nutrition Therapy, ~60 students
Instructors of Record: Donna Zoss, MS, RD; Kathleen Hill Gallant, PhD, RD

STUDENT MENTORING AND SUPERVISORY EXPERIENCE

Hill Gallant Laboratory
Trained, scheduled, and mentored two undergraduate laboratory staff members with biochemical assays and data entry

Didactic Program in Dietetics, Purdue University
Undergraduate dietetics student mentoring for future career goals
Title: Graduate Student Panel: Applying to Graduate School

ITaP (Information Technology at Purdue) Student Training Program
Student Training Schedule Coordinator June 2012 – December 2013

Wabash National Corporation
SAP Business Analyst October 2010 – January 2012

ITaP (Information Technology at Purdue) Customer Service Center
Representative/Representative Supervisor June 2006 – June 2010
Customer Service Center Summer Intern May 2009 – August 2009

SERVICE ACTIVITIES

LEADERSHIP in PROFESSIONAL ORGANIZATIONS and COMMITTEES

Moderator of Oral Session, American Society of Nutrition, Experimental Biology Meeting 2017
Student Ambassador, American Society of Nutrition, Vitamin and Mineral Research Interest Section 2016-2017
Graduate Student Representative, Purdue University Nutrition Science Alumni Network 2016-2017
President, Nutrition Science Graduate Student Organization, Purdue University 2016-2017

STUDENT VOLUNTEERISM

Purdue Child Wellness Clinic, Purdue University	October 2014
Food Finders Food Bank, Lafayette, IN	Summer 2013
Westminster Village Retirement Community	October – December 2012

PROFESSIONAL ORGANIZATION MEMBERSHIPS

Association for Clinical and Translational Science (ACTS), student member	November 2018 - Present
Women in Nephrology, student member	November 2018 - Present
Indiana Academy of Nutrition and Dietetics (IAND), student member	November 2018 - Present
Academy of Nutrition and Dietetics (AND), student member	November 2018 - Present
American Society of Nephrology (ASN), student member	October 2016 – Present
American Society of Nutrition (ASN), student member	October 2014 – Present
ASN Vitamin and Mineral Research Interest Section, Student Representative	June 2016 – June 2017
Nutrition Science Graduate Student Organization, president	September 2016 - 2017
member	August 2015 - Present
Graduate Women in Science, Omega Chapter, member	December 2015 - Present
Purdue Center for Aging and the Life Course, graduate student member	May 2016 – May 2019
Purdue Musculoskeletal and Mineral Work Group, student member	August 2015 - Present
Alpha Sigma Lambda National Honor Society, President	April 2014 – May 2015
Purdue University Nutrition Society, member	August 2013 – May 2015

ADDITIONAL TRAININGS and CERTIFICATIONS

CITI Training in Responsible Conduct of Research CITI	December 2024
CITI training for Good Clinical Practice, CITI	January 2021
CITI Training for Human Research, CITI	January 2023
CITI Training in Biosafety for Principle Investigators	December 2020
Blood-borne Pathogens and Biosafety, Purdue University	October 2020
Department of Transportation (DOT) Radiation Training, Purdue University	October 2020
Radiation Safety, Purdue University Radiological and Environmental Management	July 2020

EQUIPMENT, SOFTWARE, RESEARCH and LABORATORY SKILLS

- Writing applications to the Institutional Review Board (IRB) for human subjects research
- Recruitment and screening of research participants
- Consenting research participants
- Anthropometric measurements

- Collection, processing, and storage of human specimens (blood, urine, and feces)
- REDCap data management program
- SAS statistical software
- WINSAAM Kinetic Modeling Software