THE PHYSIOLOGICAL RELEVANCE OF THE ADAPTIVE CAPACITY OF INTESTINAL PHOSPHORUS ABSORPTION

by

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ABSTRACT

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Intestinal phosphorus absorption is a key contributor to the body phosphorus pool, but much is unknown regarding physiological adaptations in intestinal phosphorus absorption that occur in vivo. We sought to measure changes in intestinal phosphorus absorption efficiency and phosphorus balance in adolescent females and in rats in response to several factors, using physiologically relevant assessment approaches including whole-body phosphorus balance techniques and *in situ* ligated intestinal loop absorption methods.

We first assessed phosphorus balance and net phosphorus absorption in female adolescents from a controlled crossover study with two levels of calcium intake. Despite an increased calcium intake of 600 mg/day, there was no change in phosphorus balance, nor a significant change in net phosphorus absorption.

Next, we measured intestinal phosphorus absorption efficiency with the *in situ* ligated loop method in healthy Sprague Dawley rats as well as the Cy/+ rat model of progressive kidney disease. We found 10-week-old healthy rats had a small but higher absorption efficiency of phosphorus compared to 20- and 30-week-old rats, while 20-week Cy/+ rats had higher absorption efficiency than 30-week-old. Each of these results corresponded to net phosphorus absorption from balance as well as the concentration of 1,25-dihydroxyvitamin D3. In healthy rats, there was no effect of altering the level of phosphorus in the diet on absorption efficiency. In Cy/+ rats, kidney disease produced a small *increase* in absorption efficiency, contrary to the predicted decrease that would occur with lower 1,25-dihydroxyvitamin D3 observed in CKD. Gene expression of the major intestinal phosphate transporter, NaPi-2b, largely followed absorption patterns.

The utility of the Cy/+ model is limited to males as females do not begin to show signs of progressive kidney decline until a much older age. Therefore, we sought to test whether ovariectomy would accelerate kidney disease in Cy/+ females, with the aim of establishing a

postmenopausal model of progressive kidney disease. Our results show that kidney disease is not accelerated by ovariectomy in this rat strain, as measured by kidney weight and biochemistries including blood urea nitrogen, creatinine, creatinine clearance, and plasma phosphorus and calcium.

Our results utilizing *in situ* absorption measures as well as net absorption of phosphorus suggest that some of the factors that are understood to influence the intestinal absorption of phosphorus do not have a significant influence in a physiological context.

CHAPTER 1: INTRODUCTION

The intestinal absorption of phosphorus has been studied for nearly a century, yet there is much still unresolved about how it is regulated and the importance and extent of its adaptive capacity. Strategies for the treatment of chronic kidney disease (CKD) include limiting the absorption of dietary phosphorus, and the optimization of such therapies may be improved with a better understanding of intestinal transport. This chapter will overview our current knowledge of phosphorus as a nutrient, its hormonal regulators in the body, how it is transported through the kidney and intestine, how various factors may change this transport, and why this is relevant to kidney disease. Subsequent chapters of this dissertation present our work that focuses on understanding how four such factors affect the intestinal absorption of phosphorus: calcium intake in adolescent females, amount of phosphorus in the diet, age, and kidney disease progression in rats. Finally, we studied whether ovariectomy would accelerate kidney decline in a female model of CKD to enable us to study these factors in both sexes.

Roles and Sources of Phosphorus

Phosphorus is an essential mineral in human health. In humans, approximately 85% of phosphorus is contained in teeth and bone, largely in the form of hydroxyapatite. The other 15% is in located within soft tissue (1). Of this, phosphorus in the blood totals approximately 40 mg/dL, with the normal range of plasma inorganic phosphate at 2.5-4.5 mg/dL (2). Phosphorus plays diverse roles within these compartments; it plays a large structural role in bone as hydroxyapatite, in cell membranes in phospholipids, and in the sugar phosphate backbone of nucleic acids. Other roles include acid-base balance, where phosphorus serves as an important intracellular buffer; energy metabolism, within transport (e.g. ATP) and storage (e.g. creatine phosphate) forms of fuels; and cellular signaling (e.g. phosphorylation) (1).

Dietary phosphorus is widespread in the Western food supply and is present in organic and inorganic forms. While the RDA for phosphorus is set at 700 mg/day for adults (3), data from the National Health and Nutrition Examination Survey (NHANES) show that the average

intake of phosphorus in U.S. adults over the age of 20 years is about twice the RDA at 1399 mg/day based on nutrient database values (4). It is estimated that total phosphorus intake has risen over recent decades through the 1990s (5), and has been relatively stable between 2001 and 2014 (4).

The bioavailability of dietary phosphorus varies between and within organic and inorganic sources. While the estimated absorption of organic phosphorus is ~50-60%, absorption of inorganic phosphate is much more efficient, estimated at over 90% (6). However, as an important aside, many of these estimates come from methods that more accurately reflect bioaccessibility (availability to be absorbed) versus bioavailability (absorbed and available to the body tissues). Organic phosphorus exists as complex molecules within cell membranes and tissues, such as phytate in plants and phosphoproteins (e.g. casein) in animal tissues. Grains account for ~ 29% of total intake, milk and milk products ~21%, and meat, poultry, fish and mixtures ~25% (4). Thus, dietary intake of organic phosphorus is highly correlated with dietary protein intake (7), and it is difficult to limit intake of one and not the other. In contrast, inorganic phosphorus in the diet comes primarily from phosphate additives used in food processing and is not associated with protein content in these food sources. Substitution of foods with phosphate additives has been shown to potentially contribute up to 1000 mg in additional dietary phosphorus per day (8), which may have long-term negative effects on bone and the cardiovascular system (9-11).

In addition to wide ranging bioavailability of various food sources of phosphorus, assessment of dietary phosphorus intake is challenged by incompleteness and inaccuracies of the available nutrient databases. This has been shown in a number of studies that have compared estimated phosphorus in foods or meals as listed in nutrient databases to measured phosphorus and demonstrate a wide range of underestimation of phosphorus content spanning ~15-70% (12-17). Therefore, care and caution should be taken when estimating phosphorus intake based on database values.

Hormonal Regulation of Phosphate Homeostasis

The homeostatic regulation of phosphate is controlled primarily by three hormones produced by the kidney (1,25-dihydroxyvitamin D, 1,25D), bone (fibroblast growth factor-23,

FGF-23), primarily from osteoclasts but also osteoblasts), and parathyroid glands (parathyroid hormone, PTH). To the best of current knowledge, transient elevations in serum phosphate are indirectly sensed when ionized serum calcium complexes with phosphate, decreasing extracellular calcium concentrations. This results in the elevation of PTH mRNA stability and increased PTH secretion. Elevated serum phosphate can also increase PTH secretion independent of changes in ionized or total calcium (18-22). PTH binds its receptor, PTHR1, in the renal proximal tubule and signals the internalization of intramembranous sodium-phosphate cotransporter proteins, NaPi-2a and NaPi-2c, leading to a reduced reabsorption of phosphate and greater urinary phosphate excretion. In the bone, chronic PTH signals via osteoblasts and the RANK-L pathway to increase osteoclastic bone resorption. This releases phosphate (and calcium) from the bone mineral. In the kidney, PTH increases 1-alpha-hydroxlase in the proximal tubule which hydroxylates 25-OHD to the active form 1,25D. A study in healthy humans demonstrated this relationship by injecting PTH and observing an elevated 1,25D (and also FGF-23) (23). The main action of 1,25D is to increase intestinal calcium absorption but also phosphorus absorption. However, the net result of elevated PTH is a reduction in serum phosphate from its effect on increased renal phosphate excretion. Thus, in phosphate homeostasis, PTH is first and foremost a phosphaturic hormone.

1,25D acts at the intestine to increase phosphate absorption and also increase bone formation and resorption. However, because a high calcium and phosphorus diet can rescue impaired mineral homeostasis and the bone phenotype in vitamin D receptor (VDR)-null mice, it suggests that the primary role of 1,25D is in the intestine (24). 1,25D also completes a feedback loop to inhibit PTH synthesis and secretion (25). Injection of 1,25D has been shown to increase FGF-23 in mice and humans (26, 27). FGF-23 is a powerful phosphaturic hormone and participates in feedback loops with both 1,25D and PTH. FGF-23 binds to the FGFR1 receptor with the Klotho co-receptor in the distal tubule of the kidney, where it decreases NaPi-2a and NaPi-2c in the proximal tubules by an unknown mechanism (possibly paracrine signaling or an unknown signal-transduction pathway) (25). This results in decreased reabsorption of phosphate in the kidney and increased urinary phosphate excretion. As stated above, 1,25D increases FGF-23. Interestingly, dietary phosphate in a high amount (~2500 mg/d) also increases FGF-23 in humans independent of 1,25D (28), but this vitamin D-independent signaling mechanism is currently unknown. FGF-23 also provides negative feedback on 1,25D by reducing expression of

the CYP271b gene which codes for the 1-alpha-hydroxylase enzyme in the kidney to reduce the production of active 1,25D. PTH and FGF-23 are also in a negative feedback loop where PTH increases FGF-23 and FGF-23 decreases PTH (**Figure 1.1**).

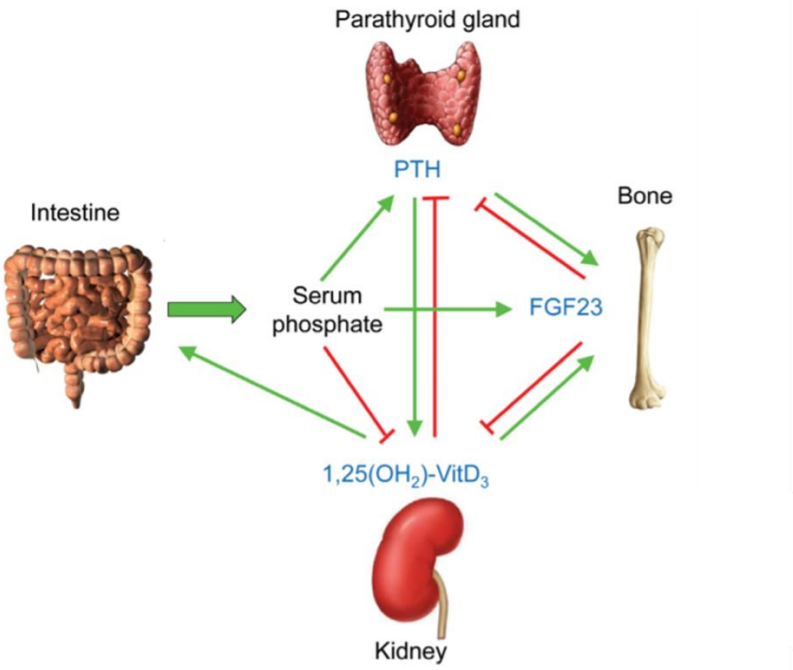

Figure 1.31. Hormonal regulation of phosphorus. *Reprinted with permission from John Wiley and Sons* (29).

The importance of these hormones has been demonstrated in human disease as well as in transgenic, knockout, and disease models in animals. A number of human genetic disorders have been identified in genes related to these hormones that support their characterized roles in phosphate homeostasis. For example, FGF-23 was first recognized as an important phosphate regulator when missense mutations that result in a reduction in FGF-23 cleavage and increase in circulating levels were identified as the cause of autosomal dominant hypophosphatemic rickets, a rare genetic disease characterized by phosphate wasting and bone demineralization (30). Another example is tumoral calcinosis type 3 which is characterized by deactivating mutations in FGF-23 that results in hyperphosphatemia and high 1,25D (25). Transgenic mice overexpressing FGF-23 also show hypophosphatemia along with phosphate wasting and a reduced expression of

NaPi-2a (31). Similarly, VDR-null mice develop secondary hyperparathyroidism and hypophosphatemia, largely due to an inability to absorb dietary calcium and phosphate (32).

Evidence for the Presence of Currently Unknown Phosphate Regulators

It has been postulated that a yet unknown phosphatonin(s) are responsible for rapid adaptations of renal excretion of phosphorus in response to changing dietary intakes that is independent of known regulators. This is supported by a rat study that infused sodium phosphate or sodium chloride into the intestinal lumen over a 30 minute period and demonstrated a rapid increase in urinary phosphate, without a change in serum phosphate, PTH, FGF-23, or sFRP-4, and also occurring with renal denervation (33). However, the study used a supraphysiologic phosphorus load of 1.3 M (34). When a 1.3 mM load was tested, the effect was not reproduced (35). Further, Martin and colleagues have shown a rapid increase (5 minutes) in PTH in response to duodenal infusion of phosphonoformate (22). Additionally, in healthy humans, IV or duodenal infusion of phosphate over a 36 hour period caused an increase in plasma phosphate concentrations and PTH followed by an elevation in urinary phosphate, and later FGF-23 and a reduction in 1,25D (18). This temporal response supports known mechanisms of phosphate regulation. Most recently, Thomas and colleagues showed that rapid phosphaturic responses elicited by intravenous or intragastric phosphate load are consistent with changes in PTH, as parathyroidectomy prevented a phosphaturic response (36). Another phosphatonin, matrix extracellular phosphoglycoprotein (MEPE), was shown by Marks et al. to also affect intestinal phosphorus absorption after a 3 hour infusion in rats (37). Using an in situ ligated loop method to measure absorption, they found that MEPE stimulated absorption in the duodenum independent of changes in serum PTH, 1,25D, or FGF-23. Further studies are necessary to evaluate the effect of MEPE on NaPi-2b and other known sodium-dependent phosphorus transporters in response to dietary phosphorus manipulations. Thus, a direct acute signal from the intestine to induce phosphaturia isn't strongly supported; PTH remains the likely candidate that mediates these fast responses.

Alterations in Phosphorus Homeostasis in Chronic Kidney Disease-Mineral Bone Disorder

The relationship of these phosphate regulatory hormones is well-demonstrated in CKD, where a reduced ability to excrete phosphate in the urine results in an increase in serum

phosphate concentrations, though this is not clinically detectable until very late in kidney failure because of the ensuing hormonal cascade that maintains homeostasis. CKD-Mineral Bone Disorder (CKD-MBD) is characterized by abnormalities in laboratory values related to calcium and phosphate homeostasis, bone abnormalities, and vascular and other soft tissue calcifications (38). As kidney function declines, FGF-23 first increases and is followed by a reduction in 1,25D, and an increase in PTH in temporal sequence as renal function declines (39). Although PTH and FGF-23 are both elevated in CKD and have opposing actions on the 1-alpha-hydroxylase enzyme, 1,25D concentrations fall in both humans and animal models (2, 40), indicating the strength of FGF-23 relative to PTH regarding their effects on 1,25D. Until the discovery of FGF-23 and its functions, the decline in 1,25D with kidney disease was thought to occur because of decreased kidney function including that of the 1-alpha-hydroxylase enzyme. However, it is now known that the direct action of FGF-23 to suppress conversion of 25D to 1,25D via the 1-alpha-hydroxylase enzyme is instead responsible. PTH is elevated in kidney disease as a result of signaling through increased serum phosphate and subsequently lower serum ionized calcium (as described above), and through the removal of the suppressive effect of 1,25D on PTH. Thus, the overall purpose of the alterations in the phosphate-regulating hormones in CKD-MBD are to increase urinary phosphate excretion via the actions of the phosphaturic hormones FGF-23 and PTH, and decrease intestinal phosphate absorption through decreased levels of 1,25D. Unfortunately, these alterations are not benign, nor are they sufficient to stave off hyperphosphatemia in later kidney failure. The off-target effects include but are not limited to increased bone resorption as a result of elevated PTH and lower calcium absorption due to decreased 1,25D, as well as increased left ventricular hypertrophy as a result of increased FGF-23. Vascular calcifications in turn are increased due to hyperphosphatemia and are potentially exacerbated by pharmacologic efforts to decrease PTH (such as through calcium loading by calcium-based phosphate binders, or through calcitriol or active vitamin D analogs). The clinical consequences of CKD-MBD are increased risk of bone fragility fractures, cardiovascular events, and death. In fact, fractures are ~4 times more prevalent in end-stage kidney disease patients compared with the general population (41), and cardiovascular mortality (not kidney failure) is the leading cause of death in patients with CKD (42).

Renal Reabsorption of Phosphorus

An introduction to mechanisms of renal reabsorption of phosphorus is helpful in understanding intestinal absorption of phosphorus. This is because the renal mechanisms were identified first and have been more fully elucidated, and because notable similarities as well as differences exist between renal reabsorption and intestinal absorption mechanisms.

The kidney is the central point in the regulation of phosphorus balance in response to changing dietary intake. In the rat, renal phosphorus reabsorption is upregulated within 2-4 hours of dietary phosphorus restriction (43, 44), while oral administration of phosphorus promotes renal excretion in under one hour (33, 45). When renal mass is reduced, there is a delay in the renal adaptation to a low phosphorus diet, but in the 5/6 nephrectomy rat model plasma phosphate can be maintained until renal function declines to less than 20% of normal (46).

The majority of renal reabsorption (~80%) occurs in the proximal tubules by a sodium-dependent process (47). Sodium-dependent phosphate transport in the kidney is mediated by three families of transporters: type I, type II, and type III, which correspond to genes of the solute carrier series SLC17, SLC34, and SLC20 (48). The two major transporters in the kidney, sodium-dependent phosphate co-transporter type II a and c [NaPi-2a (SLC34A1) and NaPi-2c (SLC34A3)], were discovered through expression cloning (49-51). Both are present at the brush border membrane (BBM) in proximal tubule cells but are absent in other nephron segments. More recently, a transporter of SLC20 family, PiT2 (SLC20A2) was identified in the proximal tubule, although at a low expression (47, 52) (**Figure 1.2**).

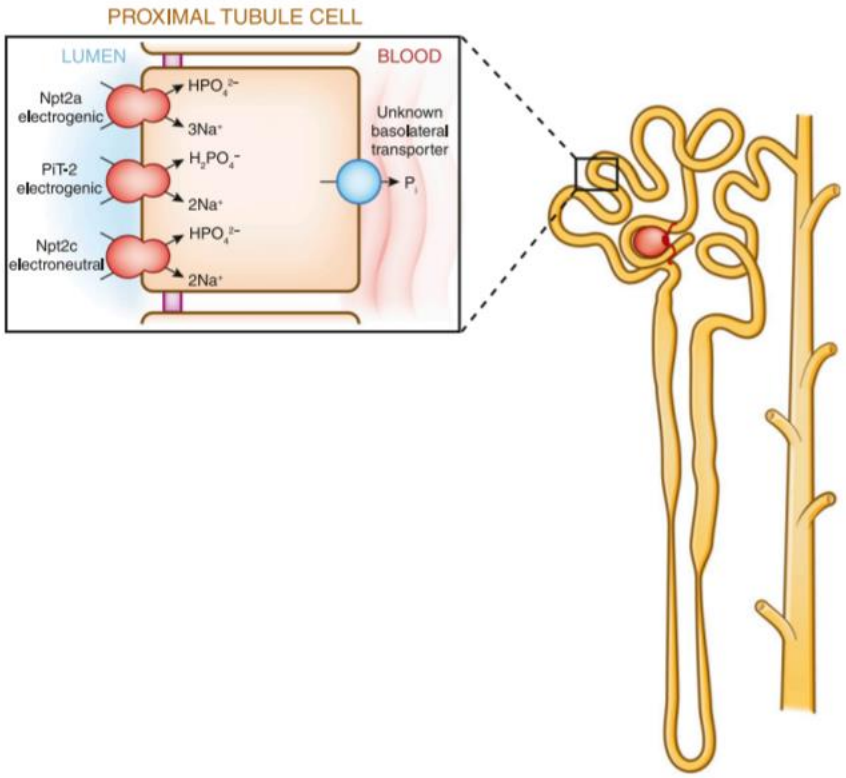

Figure 1.2. Renal phosphate transporters. *Reprinted with permission from the American Society of Nephrology* (53).

Intestinal Absorption of Phosphorus

Intestinal phosphorus absorption occurs by both sodium-dependent transcellular and sodium-independent paracellular pathways primarily in the proximal small intestine in humans and in rats. In contrast to phosphorus transport in the kidney, which is largely sodium-dependent, McHardy and Parsons (54) first showed that intestinal phosphorus absorption occurred by both pathways using an oral gavage method in rats. Many subsequent experiments have assessed the relative contributions of sodium-dependent and independent pathways in the intestine, and results vary widely with sodium-dependent contributing between ~0-80% of total transport, likely dependent on the phosphate concentration and absorption technique used (**Table 1.1**). *In situ* and *in vivo* methods likely underestimate sodium-dependency because of endogenous secretion of sodium and residual luminal phosphate concentrations, which would favor higher

passive absorption (55). The regional distribution of intestinal absorption in the intestine has been shown to occur highest in the proximal intestine in humans and rats, whereas the rate of absorption is higher in the ileum in mice (**Table 1.2**). In vivo compartmental analyses that account for both the absorption rate and transit time demonstrate that distal intestine is where ~40-46% of phosphorus is absorbed (56, 57).

The major intestinal phosphate transporter was identified as NaPi-2b (SLC34A2) using the cDNA library screening method to find transporters homologous to NaPi-2a or 2c (58, 59), and is present in the apical BBM of enterocytes (58). PiT1 (SLC20A1) and PiT2 (SLC20A2) were identified in intestinal BBM after being identified as sodium-dependent transporters in other tissues (60-62), and purportedly contribute <10% of sodium-dependent transport, although knockout models have not been performed to confirm this (**Figure 1.3**). Regional phosphate transporter distribution among species generally follows absorption patterns (**Table 1.3**).

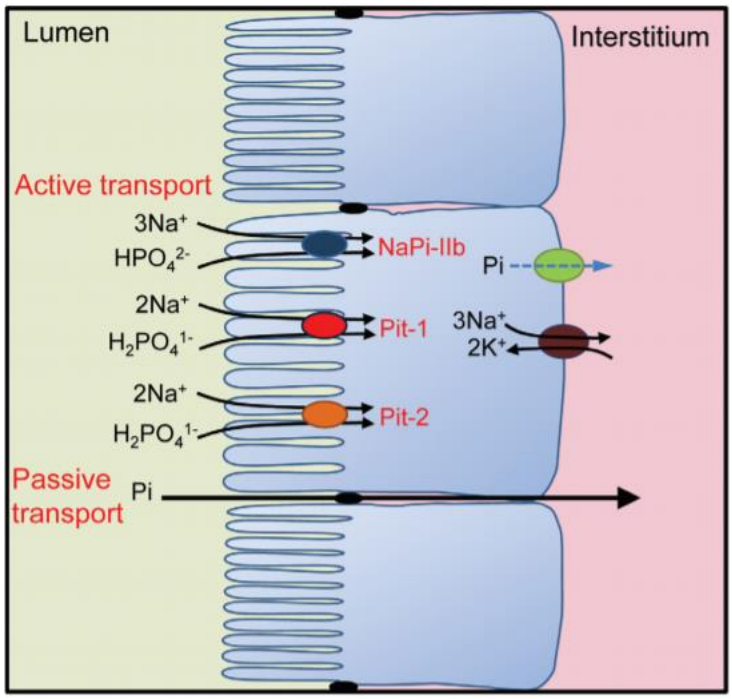

Figure 1.3. Intestinal phosphate transporters. *Reprinted with permission from John Wiley and Sons* (29).

Recent studies have further evaluated the relevance of NaPi-2b with knockout models. Ikuta et al. evaluated intestinal uptake in BBM vesicles (BBMV) and via the everted gut sac

method in NaPi-2b knockout mice and showed reduced BBMV uptake with knockout, but gut sac uptake was only significantly decreased when the transport buffer phosphate concentration was 1 mM and not 4 mM (63). This may indicate a reduced relevance of NaPi-2b as luminal concentration increases, with a higher occurrence of passive absorption. Interestingly, although NHE3, PiT1, nor PiT2 mRNA changed from NaPi-2b knockout, several claudin mRNA and protein levels were reduced, although the significance of this is unclear. The hypothesis that the active component of phosphorus absorption via NaPi-2b may be relatively most important to overall phosphorus absorption when dietary phosphorus is low is further supported by a study by Knöpfel et al. who showed that a low phosphate diet in NaPi-2b knockout mice was insufficient to maintain plasma phosphorus and resulted in bone demineralization compared to adequate to a high phosphate diet (64). NaPi-2b knockout in an adenine induced mouse model of CKD reduced serum phosphate, but FGF23 and some bone parameters were improved only when a phosphate binder was added (65). Interestingly, through manipulation of dietary phosphate, pH, and the NaPi-2b inhibitor phosphonoformate, Candeal et al. found that the changes in known phosphate transporters are unable to fully explain changes in intestinal transport, suggestive of still unknown transporters (66). The identity of basolateral transporter(s) of phosphorus is still unresolved (67).

Although renal adaptation to changes in dietary phosphorus is important to maintain phosphorus homeostasis, the intestine also plays a role. However, the relative ability of the intestine to regulate phosphate homeostasis in response to dietary changes or when kidney function fails is questionable. How intestinal P absorption is regulated has long been a subject of inquiry but has become more accessible recently with the characterization of intestinal transporters.

As discussed above, 1,25D is the phosphate-regulating hormone that has a primary role in regulating intestinal phosphorus absorption. This was first shown by Chen and colleagues in vitamin D deficiency by feeding 1.1 mg of cholecalciferol to rats on a rachitogenic diet (68). Phosphate uptake increased in the duodenum and jejunum vs control animals, as measured by the everted sac method. Many other studies injecting 1,25D have confirmed this observation in animals (68-93) and humans (94). The administration of 1,25D has also been shown to increase NaPi-2b expression in parallel with absorption. 1,25D was given intraperitoneally to mice fed a normal phosphorus diet (0.85%), and absorption measured by rapid filtration 24 hours later (95).

1,25D increased absorption efficiency and NaPi-2b protein levels but not NaPi-2b transcript levels, suggesting a post-transcriptional regulation.

In addition to the hormonal effects of 1,25D on intestinal phosphorus absorption, two important factors that have been identified by some studies that influence intestinal P absorption efficiency are 1) the level of phosphorus in the diet, and 2) age of the animal. Many animal studies since the 1970s have examined the effect of phosphorus deficiency on intestinal transport. Decreasing the level of dietary phosphorus to deficiency consistently increases sodium-dependent intestinal phosphorus uptake when measured using the rapid-filtration technique in isolated BBMV in rats (60, 66, 96, 97) and mice (95, 98-100). Further, Lee and colleagues demonstrated an increased active transport as measured by the Ussing technique in the jejunum (101). NaPi-2b gene expression tends to increase in most (52, 102-104) but not all (105-107) studies when dietary phosphorus is restricted, while NaPi-2b protein consistently increases (52, 66, 102-104, 107), suggesting post-transcriptional regulation of the transporter under certain conditions. Other transporters such as PiT1 and PiT2 are unchanged in response to dietary phosphorus restriction (52, 105) except in one study (66), which found differences in expression of these transporters depending on duration of dietary phosphorus restriction. Intestinal uptake in isolated intestinal segments may not reflect physiologic conditions. Marks et al. found no change in jejunal absorption efficiency as measured by the ligated loop in CKD rats in response to dietary phosphorus restriction (106), while Rizzoli et al. found conflicting responses in healthy female rats as measured by the ligated loop in the duodenum dependent on length of restriction and concentration of phosphorus (72).

For several reasons, it was thought that elevated 1,25D mediated the adaptive increase in active phosphate absorption in response to a low phosphorus diet. Animal studies of dietary phosphorus restriction have consistently demonstrated concurrent effects of restriction on increased 1,25D levels and phosphorus absorption. In chicks, an oral gavage method was used to assess intestinal phosphorus absorption efficiency in response to a low and normal phosphorus diet (56). Dietary phosphorus restriction caused an elevation in 1,25D concentration as well as an increase in phosphorus absorption efficiency. Danisi et al. studied rats fed a low (0.02%) compared to normal (0.6%) and found that sodium-dependent transport was increased, but this change was preceded by an increase in plasma 1,25D (57). Sruissadaporn et al. studied rats on a low phosphorus diet of 0.1% compared to a normal diet of 0.65% for 10 days, and found that

serum 1,25D levels were increased by feeding the low phosphorus diet (58). Collectively, these studies are consistent with a regulatory role for 1,25D in intestinal phosphorus absorption.

Despite increases in 1,25D in response to low phosphorus diets and increases in absorption in response to 1,25D administration, VDR knockout studies indicate that elevated 1,25D is not required for the increase in phosphorus absorption efficiency that occurs with dietary phosphorus restriction. Segawa et al. compared phosphate uptake by rapid filtration in BBMV in VDR knockout and wildtype mice on normal (0.5%) and low (0.25%) phosphorus diets (102). Although the VDR knockout reduced phosphorus uptake in mice on the normal diet, neither the adaptive increase in phosphorus uptake nor NaPi-2b levels were diminished by the loss of VDR in response to the low diet. The vitamin D independence of that adaptive increase in phosphorus absorption efficiency following dietary phosphorus restriction was confirmed by Capuano and colleagues in studies using both VDR and CYP27B1 (1-alpha-hydroxylase gene) knockout mouse models (103). In addition, these authors also found that intestinal NaPi-2b mRNA levels were increased to a similar extent when wild-type and VDR knock-out or CYP27B1 knock-out mice were fed a low (0.1%) phosphorus diet compared to a normal phosphorus diet (0.8%). Together these data suggest that neither 1,25D nor its binding to VDR are required for the increase in active phosphorus absorption that occurs during phosphorus restriction. The alternative, nongenomic signaling pathway through the membrane-associated, rapid-response, steroid-binding (MARRS) receptor has been shown to mediate 1,25D stimulated phosphate uptake (108, 109), although changes in uptake and NaPi-2b in response to phosphate restriction likely occur too slowly to be explained by this pathway. Additional candidates are required to further understand absorption regulation in this context.

Animals experience rapid growth during the early stages of life, and like other nutrients it may be expected that there is an upregulation of intestinal absorption of phosphorus to support tissue accrual. The effect of age was first studied in rodents by Borowitz and colleagues, who showed an increase in sodium-dependent and -independent transport in younger rats in the jejunum using the rapid-filtration technique (1985 cite). Other *in vitro* uptake studies have confirmed this finding in rats (Ghishan 1988; Xu 2002) and mice (Arima 2002). Armbrecht (1986) similarly showed an increased uptake in young rats in the duodenum and jejunum but not ileum in young rats using the everted gut sac method. The effects of age are further discussed in Chapter 3.

Additional factors have been studied to a lesser extent on their impact on intestinal absorption of phosphorus. Acute glucocorticoid treatment has been shown to inhibit sodium-dependent phosphate uptake in rabbits (110), and rats concurrent with a reduction in NaPi2b mRNA (111). Administration of a glucocorticoid for 7 days has been shown to increase passive absorption of phosphate in all intestinal segments except the colon in rats (112). In pigs, acute glucocorticoid administration caused an increase in absorption but chronic treatment resulted in a downregulation (113, 114). Inhibitors of protein synthesis also decrease absorption, suggesting that such proteins that have a rapid turn-over influence absorption (114). Exogenous growth hormone increased net phosphorus absorption in pigs (115) Estradiol administration in rats increased BBMV uptake via NaPi-2b mRNA/protein (116). Epidermal growth factor inhibits NaPi-2b mRNA (117). Phosphophloretin derivatives (118, 119) as well as phosphonoformic acid (66, 120, 121) and liver X receptor agonism (122) also inhibit sodium-dependent BBMV uptake. Diaminobutane dendrimers have also been explored as phosphate binders (123), while additional binders are discussed below. Metabolic acidosis increases sodium-dependent BBMV uptake (124, 125) via post transcriptional regulation of NaPi-2b (124). Finally, energy status may influence NaPi-2b regulation through changes in AMPK-mTOR via the AMP:ATP, which may be an adaptive response to turn ATP-generating and -consuming pathways on and off (126).

Table 1.1. Differences between absorption methods in estimating the proportion of intestinal sodium-dependent uptake/absorption.

			Na+ -dependent (% of total)			
Species	**Technique**	**[P] (mM)**	**Duodenum**	**Jejunum**	**Ileum**	**Ref**
Rat (SD)	Ligated loop	0.1	0	32	52	(55)
Rat (SD)	Ligated loop	10	0	29	10	(55)
Rat (SD)	Oral gavage	0.5	0 (all segments)			(127)
Rat (SD)	Everted sac	0.1	48	73	0	(55)
Rat (SD)	Everted sac	10	48	53	0	(55)
Mouse	Rapid filtration	~0.1	~50	~50	~80	(104)
Human	Rapid filtration	0.1		~40		(128)
Human	Rapid filtration	1		~30		(128)

Table 1.2. Species differences in rate of absorption of intestinal phosphate.

Species	**Technique**	**Segment with highest rate**	**2nd highest**	**3rd**	**4th**	**Ref**
Rat (SD)	Ligated loop/everted sac	Jejunum	Duodenum	Ileum	Distal colon	(55)
Rat (SD)	Ligated loop	Duodenum	Jejunum	Ileum		(91)
Rat (Holtzman)	Ussing	Jejunum	Duodenum	Ileum		(129)
Human	Triple lumen perfusion	Jejunum	Ileum			(130)
Mouse (C57BL/6)	Ligated loop	Ileum	Jejunum/duodenum			(91)
Pig	Ussing	Jejunum	Duodenum	Ileum (negligible)		(131)

Table 1.3. Species segmental differences in intestinal phosphate transporter gene and protein expression.

Species	Gene or Protein	Duodenum	Jejunum	Ileum	Colon	Ref
Rat	Slc34a2 mRNA (NaPi2b)	2	1	-		(52, 91)
Rat	NaPi2b protein	2	1	-		(52)
Rat	Slc20a1 mRNA (PiT1)	3	2	1		(52)
Rat	PiT1 protein	2	1	-		(52)
Rat	Slc20a2 mRNA (PiT2)	2	1	1		(52)
Mouse	Slc34a2 mRNA (NaPi2b)	3	2	1		(91, 104)
Mouse	NaPi2b protein	-	2	1		(91, 104)

Numbers indicate rank (1 = highest, 3 = lowest) of expression of each gene/protein.

Role of Dietary Phosphorus in Renal Disease Progression

Strictly controlling the level of dietary phosphorus has been shown to slow the progression of renal disease in both animal and human studies. In Sprague-Dawley rats with 5/6 nephrectomy, Ibels et al. showed that a low phosphorus (0.04%) compared to normal diet (0.5%) for 82 days prevented renal calcification (132). Haut and colleagues showed studied uninephrectomized, partially nephrectomized, and intact Sprague-Dawley rats on 3 different phosphorus levels (0.5%, 1%, and 2%) (133). At each increase in phosphorus, particularly in the nephrectomized rats, there was an increase in renal calcium and a higher abnormal histology. Kusano et al fed irreversible Thy1 rats, a uremic model, a low (0.3%) or normal (0.5%) phosphorus diet and observed a preservation of renal function as assessed by histopathology (134). In a related study using the same rat model and dietary phosphorus levels as used by Ibels et al. (132), Karlinksky and colleagues found that renal function, as measured by urinary creatinine, was maintained on a low phosphorus diet. As a result, 73% of rats fed the low phosphorus diet were alive after 133 days versus 8% of the rats fed diets with normal phosphorus levels (135). In humans with chronic renal failure, restricting dietary phosphorus from 900 mg/d to ~700 mg phosphorus diet reduced urinary creatinine levels (136). Barsotti and colleagues found that uremic patients consuming a 7.0 mg phosphorus per kg body weight diet had a reduced creatinine clearance after 11.3 months compared to a control group following a 12 mg

phosphorus per kg body weight per day diet (137). However, while dietary phosphorus restriction is suggested by the Kidney Disease: Improving Global Outcomes (KDIGO) organization for CKD stages 3-5 (138), there are practical considerations that may make this difficult in free living people. For example, dietary phosphorus is highly positively correlated with dietary protein (139). However, although lower protein intake reduces serum phosphate, it is associated with poorer survival in hemodialysis patients (140, 141). Additionally, a post hoc analysis of a randomized controlled trial in hemodialysis patients found that, compared to patients who consumed their normal diets, patients prescribed to phosphorus levels between 1001 and 2000 mg per day had a reduction in mortality (median follow-up = 2.3 years) (142). The difficulty of separating phosphorus and protein is compounded by the ubiquity of phosphorus in the food supply. In dialysis patients, the typical 3x weekly dialysis sessions each remove ~1,000 mg of phosphorus (143), but this is easily replaced by the average dietary intake. To only reduce phosphorus, pharmacologically targeting the intestinal absorption of phosphorus may be more effective in this condition than diet modifications alone. Currently, KDIGO guidelines suggest the use of phosphorus binders to prevent intestinal absorption and manage hyperphosphatemia in stages 3-5 of CKD. Binder development has progressed through several eras since the 1970s. The various binders and other agents used or in development to limit dietary phosphorus absorption are reviewed in Appendix A.

Role of Phosphate in Non-Skeletal Mineralization

Phosphate plays an important physiologic role in skeletal mineralization (Appendix B). But, the actions of elevated serum phosphate can also affect vascular mineralization. It is well established that there is an elevated association between cardiovascular morbidity and mortality and increasing serum phosphate levels in healthy populations (144, 145) as well as people with CKD. Associations also exist between PTH and cardiovascular disease in CKD patients (146), and FGF-23 is associated with mortality in CKD patients (147).

A possible explanation for such associations is an increased calcification mediated by the changes in these hormones. Indeed, vascular calcification is prevalent in CKD- apparent in about 30-65% of patients with stage 3-5 CKD and 50-80% in end stage, as well as different rat models of CKD (eg Cy/+, (148), 5/6 nephrectomized) (149). In rats, high PTH has been shown to be important in stimulating high phosphate-induced vascular smooth muscle cell (VSMC)

osteogenic differentiation and vascular calcification (150). Interestingly, however, in a rat model of CKD (5/6 nephrectomy), chronically neutralizing FGF-23 with an antibody decreased FGF-23 but increased aortic calcification and mortality, consequent to an increase in serum phosphate (151). This suggests that serum phosphate may play a more important role in cardiovascular outcomes through its calcifying role. Indeed, in vitro mechanistic evidence thus far suggests that the elevated phosphate itself is critical in triggering vascular calcification.

Recent studies have demonstrated the ability for high phosphate to induce the transformation of VSMCs to osteoblast-like cells in culture (152-154). Osteopontin and alkaline phosphatase expression were increased in the two studies that measured them (152, 154), markers of phenotypic change and mineralization. Calcium content of the cells was increased in one study (153). The transcription factor Cbfa1/Runx2 was also upregulated in response to elevated phosphate concentration (154), which is important to osteoblast differentiation in bone and is also induced by PTH (155). The sodium-dependent phosphate transporter PiT1 has been found to be upregulated in VSCMs exposed to elevated phosphorus in culture (153), and inhibiting PiT1 expression in human smooth muscle cells reduced sodium-dependent phosphate transport and calcification in vitro (156). This suggests that elevated phosphate transport itself initiates these changes.

After the phenotypic changes of VSMCs to osteoblast-like cells, bone alkaline phosphatase is induced and degrades the mineralizing-inhibitor pyrophosphate (155). They then become calcified similar to bone: collagen and other proteins are secreted into the intima or media, and accumulate phosphorus and calcium which mineralizes into hydroxyapatite (157). It is important to note that the fate of mineralization is not definite after phenotypic changes occur. A number of local and circulating proteins have been identified that can inhibit this process, such as fetuin-A, which is inversely associated with vascular calcification in dialysis patients (158), and osteopontin which inhibits vascular calcification (155) as demonstrated in osteopontin -/- mice that experience increased calcification (159). Another, sclerostin, is inhibited by high PTH which is increased in CKD, and together with elevated phosphate contributes to a pro-calcifying milieu (150).

In addition to hormonal changes in CKD, a recent study found increased non-renal clearance, particularly to vascular tissue, of labeled IV phosphate and calcium within 30 minutes in CKD rats compared to controls, that was independent of changes in PTH and FGF-23,

suggesting additional mechanisms of deposition are not yet understood (160). Further, high phosphate may directly alter vascular function independent of mineralization (161), and has also been shown to lead to an increase in blood pressure in healthy humans (162).

Importance of Understanding Intestinal Phosphorus Absorption

Because of the central role phosphorus plays in renal disease progression and CKD-MBD cardiovascular and bone abnormalities, it is of great importance to understand mechanisms of intestinal phosphorus absorption, including the intestine's ability to adapt to various factors including diet, age, and kidney function decline. Probing this capacity with physiological methods of measuring absorption will illuminate the relative importance of the active pathway of intestinal absorption. Further, it will provide insight into the potential and importance of currently unresolved regulators of intestinal phosphorus absorption that may be of clinical interest to the treatment of CKD-MBD.

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CHAPTER 2: PHOSPHORUS BALANCE IN ADOLESCENT GIRLS AND THE EFFECT OF SUPPLEMENTAL DIETARY CALCIUM

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Abstract

There are limited data on phosphorus balance and the effect of dietary calcium supplements on phosphorus balance in adolescents. The purpose of this study was to determine phosphorus balance and the effect of increasing dietary calcium intake with a supplement on net phosphorus absorption and balance in healthy adolescent girls. This study utilized stored urine, fecal, and diet samples from a previously conducted study that focused on calcium balance. Eleven healthy girls ages 11 to 14 years participated in a randomized crossover study, which consisted of two 3-week periods of a controlled diet with low (817 ± 19.5 mg/d) or high (1418 ± 11.1 mg/d) calcium, separated by a 1-week washout period. Phosphorus intake was controlled at the same level during both placebo and calcium supplementation (1435 ± 23.5 and 1453 ± 28.0 mg/d, respectively, $p = 0.611$). Mean phosphorus balance was positive by about 200 mg/d and was unaffected by the calcium supplement ($p = 0.826$). Urinary phosphorus excretion was lower with the calcium supplement (535 ± 42 versus 649 ± 41 mg/d, $p = 0.013$), but fecal phosphorus and net phosphorus absorption were not significantly different between placebo and calcium supplement (553 ± 60 versus 678 ± 63 versus mg/d, $p = 0.143$; 876 ± 62 versus 774 ± 64 mg/d, $p = 0.231$, respectively). Dietary phosphorus underestimates using a nutrient database compared with the content measured chemically from meal composites by ∼40%. These results show that phosphorus balance is positive in girls during adolescent growth and that a calcium dietary supplement to near the current recommended level does not affect phosphorus balance when phosphorus intake is at 1400 mg/d, a typical US intake level.

Introduction

Because of rapid growth in adolescence, the phosphorus recommended dietary allowance (RDA) for girls and boys ages 9 to 13 years is set at 1250 mg/d, nearly twice as high as the RDA of 700 mg/d for adults (1). Data from the National Health and Nutrition Examination Survey (NHANES) show that the average intake of phosphorus in this age group for girls is 1176 mg/d, and 66% of children in this age group meet the estimated average (median) requirement (2). Dietary calcium binds phosphorus in the intestine and impairs its absorption. The interaction between phosphorus and calcium in the intestine in healthy adults demonstrated that the phosphorus binding capacity of calcium carbonate and calcium acetate is approximately 45 mg phosphorus per gram calcium salt (3). Thus, calcium salts are used as phosphate binders to prevent or lower hyperphosphatemia in patients with chronic kidney disease (CKD) (4).

Limited information exists on phosphorus balance and the effect of a dietary calcium supplement on phosphorus retention during adolescent growth to inform the phosphorus Dietary Reference Intakes (DRI) (5-7). This deficit in knowledge is particularly important because it is estimated from NHANES data that 24% of females ages 9 to 13 years use supplemental calcium (8).

The aims of this study were to describe phosphorus balance in adolescent girls and determine the effect of a dietary calcium supplement on phosphorus skeletal retention using stored samples from a previous study that examined the effect of particle size of calcium supplementation on calcium balance (9). In addition, because of the uncertainty of the accuracy of dietary phosphorus intake from food composition tables, we tested the relationship between dietary intake estimated from food composition tables and from chemical analyses of the diet during the balance study.

Materials and Methods

Subjects and Study Design

Stored samples from adolescent girls who participated in calcium balance studies during the summer of 2007 were analyzed for phosphorus content. A detailed description of the original study is described elsewhere (9). Briefly, healthy adolescent girls, ages 11 to 14 years, participated in a randomized crossover study that consisted of two 3-week balance studies, separated by a 1-week washout period to compare calcium balance in subjects when they were

given small particle-size calcium carbonate supplements, large particle-size calcium carbonate supplements, or a placebo. The original study found no difference in calcium balance between small and large particle-size calcium carbonate but significantly greater positive calcium balance from small particle size than placebo (9). Eleven of the 12 participants who were in the study arm that compared small particle-size calcium carbonate with placebo are included in the present analysis (Table 2.1). One participant was excluded from the present analysis because of insufficient stored fecal sample. Two of the 11 participants included completed only one of the two crossover periods. Race and ethnicity were self-reported by questionnaire. Height and weight were measured with a stadiometer and scale and used to calculate height-for-age, weight-for-age, and body mass index (BMI)-for-age percentiles from the Centers for Disease Control (CDC) growth charts using the Statistical Analysis Software (SAS) files available online from CDC (10). Sexual maturation stage was determined by breast development using a Tanner Sexual Maturity Form by self-assessment (11). The balance studies were conducted in a controlled environment in the form of a summer camp. Participants were fed a controlled diet (containing ~800 mg/d calcium) and randomized to receive either an additional 600 mg/d of elemental calcium from calcium carbonate capsules or placebo (Fig. 2.1). The controlled diet consisted of a 4-day cycle menu with consistent phosphorus, calcium, sodium, and protein content. Participants were allowed to consume deionized water *ad libitum*. Diets of differing energy content (1300 kcal/d, 1600 kcal/d, and 1900 kcal/d) were designed to meet the energy requirements of the participants as estimated by the Harris-Benedict equation (12), and weekly body weights were monitored for weight maintenance. During each 3-week balance study, all fecal and urine samples were collected, and participants were closely monitored for diet, fecal, and urine collection compliance. Duplicate diet composites were made at the time of each meal, pooled by 24 hours, and frozen. Thawed composites were homogenized, freeze-dried (FTS Systems Inc., Stone Ridge, NY, USA), and stored for later analysis. Any uneaten food was offered again to participants during the same 24-hour period, and complete intake was encouraged. Uneaten food at the end of the 24-hour period was saved, weighed, and analyzed to determine accurate intake. Polyethylene glycol (PEG), a nonabsorbable fecal marker, was provided with each meal (PEG E3350, Dow Chemical Co., Midland, MI, USA, prepared in capsules by Delavau LLC, Philadelphia, PA, USA). Pill counts, urine creatinine, and fecal PEG recovery were used as compliance measures as described previously (9).

Measures

Dietary, fecal, and urine phosphorus content

Dietary phosphorus was estimated from study menus using Nutrition Data System for Research 2007 (NDSR, Nutrition Coordinating Center [NCC], University of Minnesota, Minneapolis, MN, USA), and stored diet composite samples were analyzed for phosphorus. Freeze-dried diet and thawed fecal homogenates (stored at −20°C) were ashed in a muffle furnace (Thermolyne Sybron Type 30400, Dubuque, IA, USA) at 600°C for 3 days. Ashed diet and fecal samples were diluted with 2% nitric acid. Acidified urine samples (stored at −40°C) were thawed and diluted with 2% nitric acid. Phosphorus was measured in diet, fecal, and urine samples by inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT, USA). Daily urinary phosphorus excretion was adjusted based on average daily creatinine excretion (9) for each participant to correct for timing and incomplete sample collection errors.

Phosphorus balance and net absorption calculations

The first week of each 3-week study was used as an equilibration period to the calcium intake level, and balance calculations were based on the last 2 weeks of each 3-week study. For each balance period, balance, net absorption, and percent net absorption were calculated. Balance is calculated as dietary phosphorus intake (mg/d) minus urine and fecal phosphorus excretion (mg/d); net absorption as phosphorus intake (mg/d) minus fecal excretion (mg/d); and percent net absorption as net absorption (mg/d) divided by dietary intake (mg/d) × 100.

Statistical Analysis

Repeated measures ANOVA for crossover designs using the PROC MIXED procedure with subject as a random effect was used to compare treatment differences and included analysis for order and period effects. Unpaired *t*-tests were used to compare estimated and analytically measured dietary phosphorus and calcium. Statistical significance was set at $\alpha < 0.05$. Statistical Analysis Software (SAS Institute, Cary, NC, USA) version 9.3 was used for all statistical analysis. Results are reported as mean ± SEM unless otherwise indicated.

Results

The majority of girls were of white race and non-Hispanic ethnicity (Table 2.1). Mean BMI-for-age was greater than the 50th percentile. All were healthy and were recruited from Indiana, Illinois, and Ohio.

Phosphorus measured chemically in the daily diet samples from the placebo phase and from the calcium carbonate was 39% and 40% greater than the phosphorus content estimated by NDSR ($p < 0.001$), respectively. Calcium measured in the diet samples was not different from estimated calcium content from NDSR in the placebo phase and was 2% higher than estimated on the calcium phase ($p = 0.013$, Table 2.2). Dietary phosphorus intake measured chemically did not differ between placebo and calcium ($p = 0.611$, Table 2.2), and calcium intake was significantly different by design between placebo and calcium phases.

Overall phosphorus balance was not different between calcium and placebo (245 ± 81 mg versus 228 ± 79 mg; $p = 0.826$, NS) (Fig. 2.2). Fecal phosphorus was not significantly different between calcium and placebo (678 ± 63 mg versus 553 ± 60 mg; $p = 0.143$, NS), whereas urinary phosphorus was 114 mg/d lower with calcium than with placebo (535 ± 42 mg versus 649 ± 41 mg, $p = 0.013$). Net phosphorus absorption was not significantly different on calcium compared with placebo expressed as either mg/d (774 ± 64 mg/d versus 876 ± 62 mg/d; $p = 0.231$, NS) (Fig. 2.3*A*) or as percent of intake (53 ± 4% versus 61 ± 4%; $p = 0.186$, NS).

As previously reported (9), calcium balance was 307 mg/d higher on calcium compared with placebo, (519 ± 48 mg/d versus 212 ± 46 mg/d; $p = 0.002$), fecal calcium was higher (857 ± 56 mg/d versus 517 ± 54 mg/d; $p = 0.001$), whereas urinary calcium was not significantly different (114 ± 26 mg/d versus 84 ± 26 mg/d; $p = 0.079$, NS) (Fig. 2.4). Net calcium absorption was higher on calcium compared with placebo (569 ± 55 mg/d versus 301 ± 53 mg/d; $p = 0.004$) (Fig. 2.3*B*).

Serum calcium, phosphate, 25OHD, $1,25(OH)_2D_3$, parathyroid hormone (PTH), osteocalcin, alkaline phosphatase (ALP), bone alkaline phosphatase (BAP), and creatinine-corrected urinary N-terminal telopeptide (NTx) and free deoxypyridinoline (DPD) were not different between the placebo and calcium (Table 2.3). Values were within normal ranges on both placebo and calcium supplement.

Discussion

In this 3-week randomized, placebo-controlled crossover balance study, adolescent girls were in positive phosphorus balance of about 200 mg when consuming just over 1400 mg of phosphorus per day. Because bone contains approximately 85% of the body's phosphorus stores (13), the bulk of the retention is presumed to be in the skeleton, reflecting the high rate of skeletal growth during adolescence. A calcium supplement of 600 mg/d had no effect on this phosphorus retention but did increase the calcium retention by more than 300 mg/d. In girls ages 9 to 13 years, average estimated calcium intake from the diet is 968 mg/d (8), and thus supplements may be used to achieve the calcium RDA of 1300 mg/d (14). The calcium supplement affected phosphorus metabolism in the expected direction by decreasing urinary phosphorus, but the decrease in net phosphorus absorption was not statistically significant. This may be attributable to the higher variability in fecal phosphorus measurements compared with urinary phosphorus.

Urinary phosphorus decreased by 1.9 per 10 mg/d increase in elemental calcium intake in the calcium carbonate period. In comparison, we previously published a study of similar design in moderate-stage CKD patients, which showed that there was about 1 mg/d reduction in urinary phosphorus per 10 mg/d increase in elemental calcium (15). In the CKD study, urinary phosphorus fell with calcium carbonate supplementation, but there was no increase in fecal phosphorus, net phosphorus absorption, or overall phosphorus balance. A study of predialysis CKD patients also observed a ∼1 mg/d reduction in urinary phosphorus per 10 mg/d calcium in patients receiving ∼800 mg elemental calcium (16). The difference in reduction in urine phosphorus with supplemental calcium between our study in healthy adolescents and the CKD studies probably reflects increased efficiency to retain phosphorus in adolescents compared with adults with CKD. However, the lower amount of the calcium supplement in the adolescent study, which provided only ∼600 mg/d, may also be a factor.

There are a limited number of phosphorus balance studies in adolescent girls using different levels of phosphorus and calcium intake. Nearly 100 years ago, Sherman and colleagues (7) performed a series of balance studies in 9- to 13-year-old girls. Calcium intakes ranged from 425 to 1794 mg/d with phosphorus intakes ranging from 886 to 2009 mg/d, and phosphorus balance ranged from −37 to 667 mg/d. Ca:P intake (mass) ratio ranged from 0.48 to 1.03, and there is no relationship between Ca:P intake ratio and phosphorus or calcium balance.

More than 50 years later, Greger and colleagues (5) provided 12.5- to 14.5-year-old girls a diet of 1.07 g/d calcium and 0.85 g/d phosphorus and showed that phosphorus balance was positive (48 ± 76 mg/d), as was calcium balance (409 ± 61 mg/d). In a separate study by Greger and colleagues (6), phosphorus balance was similar to their previous study at 23 ± 110 mg/d. However, the DRIs for phosphorus for ages 9 through 13 years were set based on estimated phosphorus intake to support observed tissue accretion rather than intakes for maximal retention because published studies lacked a range of phosphorus intakes to establish maximal retention as was available for calcium (1). Our study provides additional data at an intake level intermediate to other studies (~1.4 g/d phosphorus), but studies over a wider range of phosphorus intakes will be required to determine the intake that achieves maximal retention.

Because 99% of the body's calcium (17) and 85% of the body's phosphorus (13) reside in bone as hydroxyapatite and assuming that during a 3-week balance period retained calcium and phosphate are deposited in the skeleton as apatite crystal, the relationship between calcium retention and phosphorus retention measured by balance should mirror the 2.15:1 mass ratio (5:3 molar ratio) of calcium to phosphorus in bone hydroxyapatite. On placebo, the mass ratio of the mean calcium balance to mean phosphorus balance is 1.08 and on calcium supplement is 2.47, which do not agree with the hydroxyapatite 2.15 ratio for placebo but are close for the calcium supplement. However, apart from two apparent outliers (one with very high calcium balance on the calcium supplement but with no change in phosphorus balance, and one with very negative phosphorus balance on the calcium supplement), the individual subjects demonstrate that they do generally follow a slope similar to the expected ratio line (Fig. 2.5), although the variation is high and some individuals appear to be higher or lower mineral retainers. This variation probably represents cumulative errors in the balance technique and differences in retention from natural variations in adolescent bone and soft tissue growth rates. It probably also reflects the fact that calcium phosphate is initially deposited in bone with a wide calcium to phosphate ratio (18).

The observation in our study of an underestimation of dietary phosphorus by approximately 40% in mixed meals is consistent with other studies, which have found underestimation of phosphorus content in nutrient databases spanning a wide range from ~15% to 70% (19-24). Recently, Carrigan and colleagues (21) designed 4-day menus to be low or high in phosphorus additives based on the absence or presence of phosphorus additives on food label ingredient lists, then analyzed these diets for phosphorus content and compared measured values

with the estimated values from NDSR software and reported that NDSR underestimated phosphorus in these diets by ∼14%. Other studies have evaluated the accuracy of nutrient database values for various meat products that list phosphate additives in the ingredients compared with those that do not. Sullivan and colleagues (22) analyzed the phosphorus content of 38 chicken products and found on average a 43% underestimation compared with the expected values. Benini and colleagues (23) measured an average ∼70% more phosphorus than estimated in ham, roast breast turkey, and roast breast chicken products from Italy. In our study, foods with phosphate additives listed on the ingredient label were matched as closely as possible to items in NDSR that also listed additive phosphate as an ingredient. It is clear that this method is insufficient to accurately estimate phosphorus content in mixed meals. To illustrate the potential impact of this error, we substituted the estimated dietary phosphorus from the nutrient database analysis in the balance calculations in this study. Doing so resulted in calculations of phosphorus balance of −170 mg/d and −163 mg/d for the calcium carbonate and placebo periods, respectively. Without direct chemical analysis of the phosphorus content of the foods used in our balance studies, we would have erroneously concluded that our subjects were in negative phosphorus balance on both placebo and calcium supplement conditions. This underscores the importance of chemical analysis of diet composites in phosphorus balance studies.

This crossover balance study demonstrates that adolescent girls are in positive phosphorus balance of an average 200 mg/d on a diet of ∼800 mg/d calcium and ∼1400 mg/d phosphorus. When calcium intake is increased from ∼800 mg/d to ∼1400 mg/d with a calcium carbonate supplement, phosphorus balance is unchanged despite a more positive calcium balance. Increasing dietary calcium levels within a normal dietary range (from typical intake level to around the RDA level) does not negatively impact phosphorus balance when phosphorus intake is at a level typically consumed in the United States. In addition, we confirm the need to improve estimations of phosphorus in foods in nutrient databases.

Table 2.1. Baseline Participant Characteristics

White/Asian, *n*	10/1
Hispanic/other, *n*	1/10
Age, years	13.5 ± 0.98 (11.3–14.6)
Height-for-age, percentile	48.9 ± 29.1 (6.9–97.9)
Weight-for-age, percentile	60.1 ± 32.8 (2.9–98.9)
BMI, kg/m^2	21.3 ± 2.8 (19.4–27.2)
BMI-for-age, percentile	62.3 ± 34.2 (6.2–97.1)
Tanner stage, breast, *n* for stages 1–5[a]	0/2/3/2/2

BMI = body mass index.
Mean ± SD (min-max), unless otherwise indicated.
[a] Data are missing for 2 participants.

Table 2.2. Estimated and Measured Dietary Phosphorus and Calcium

	Estimated phosphorus (mg)	**Measured phosphorus (mg)**	**Estimated calcium (mg)**	**Measured calcium (mg)**
Diet with placebo	1031 ± 5.6	1435 ± 23.5**	784 ± 0.05	817 ± 19.5
Diet with calcium carbonate	1037 ± 7.4	1453 ± 28.0**	1384 ± 0.07$^{\#}$	1418 ± 11.1*$^{\#}$

Mean ± SEM.
*$p < 0.05$ measured calcium versus estimated calcium; **$p < 0.001$ measured phosphorus versus estimated phosphorus; $^{\#}p < 0.001$ diet with placebo versus calcium carbonate.

Table 2.3. Hormone and Bone Metabolism Markers on Placebo and Calcium Supplement

	Placebo	**Calcium**	***p* Value**
Serum Ca (mmol/L[a])	2.275 (0.02)	2.28 (0.02)	0.764
Serum P (mmol/L[a])	1.49 (0.05)	1.42 (0.05)	0.208
25OHD (nmol/L)	62.47 (4.7)	69.09 (5.0)	0.256
$1,25(OH)_2D_3$ (pmol/L)	131.45 (8.9)	122.86 (8.9)	0.197
PTH (pmol/L)	2.55 (0.29)	2.40 (0.31)	0.723
Serum osteocalcin (µg/L)	21.71 (3.6)	24.58 (3.7)	0.494
Serum BAP (µg/L)	73.39 (10.4)	81.25 (10.5)	0.120
Serum ALP (IU/L)	171.34 (23.8)	172.11 (24.0)	0.958
Urinary NTx/Cr (nmol BCE/mmol)	403.79 (99.0)	410.26 (100.1)	0.916
Urinary free DPD/Cr (nmol/mmol)	20.69 (3.7)	20.6 (3.7)	0.966

Ca = calcium; P = phosphorus; PTH = parathyroid hormone; BAP = bone alkaline phosphatase; ALP = alkaline phosphatase; NTx = N-terminal telopeptide; BCE = bone collagen equivalents; DPD = deoxypyridinoline; Cr = creatinine.
Values are presented as mean (SEM).
[a] To convert to mg/dL, divide Ca by 0.25 and P by 0.323.

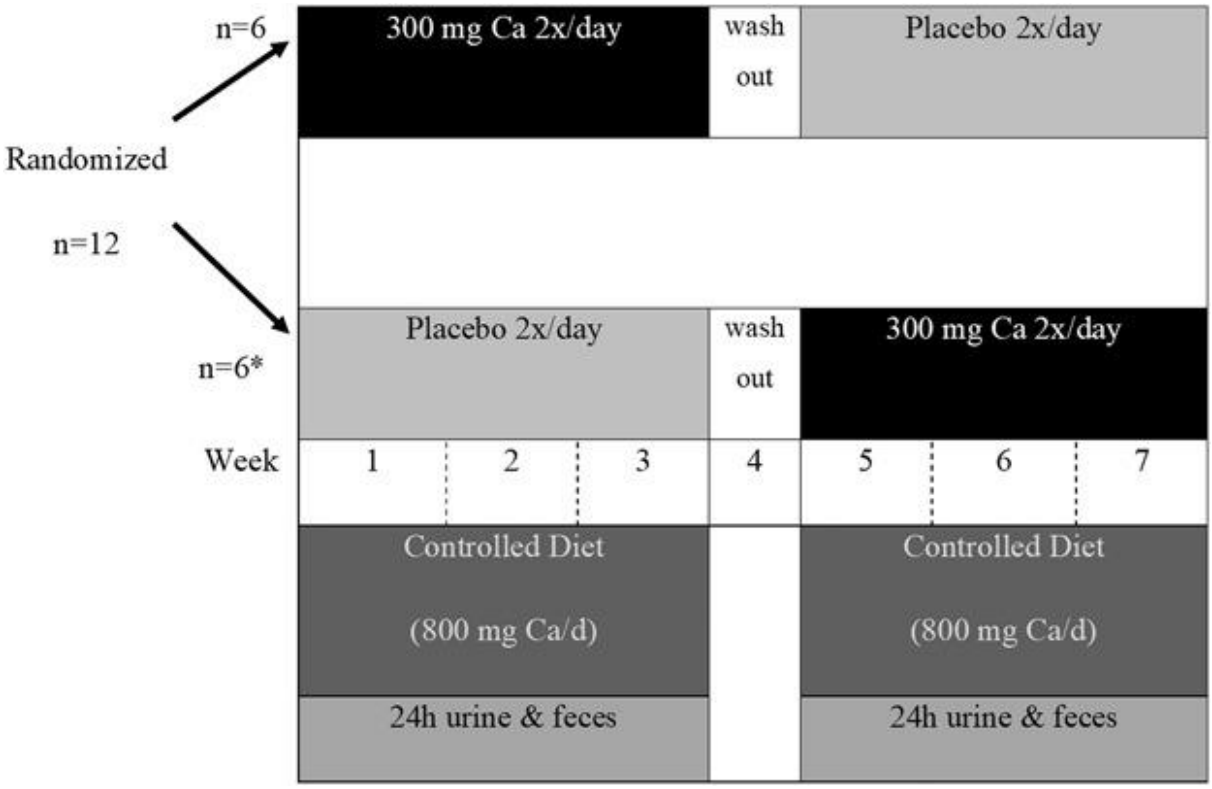

Figure 2.1. Randomized crossover study design.

Twelve participants enrolled in the study. *One participant was randomized to the placebo-calcium sequence but did not start until the second phase so did not receive the placebo; 1 participant randomized to placebo-calcium sequence did not complete the second phase of the crossover; 1 participant randomized to the placebo-calcium sequence is excluded from the present analysis because of insufficient stored fecal sample. Thus, 11 are included in this analysis.

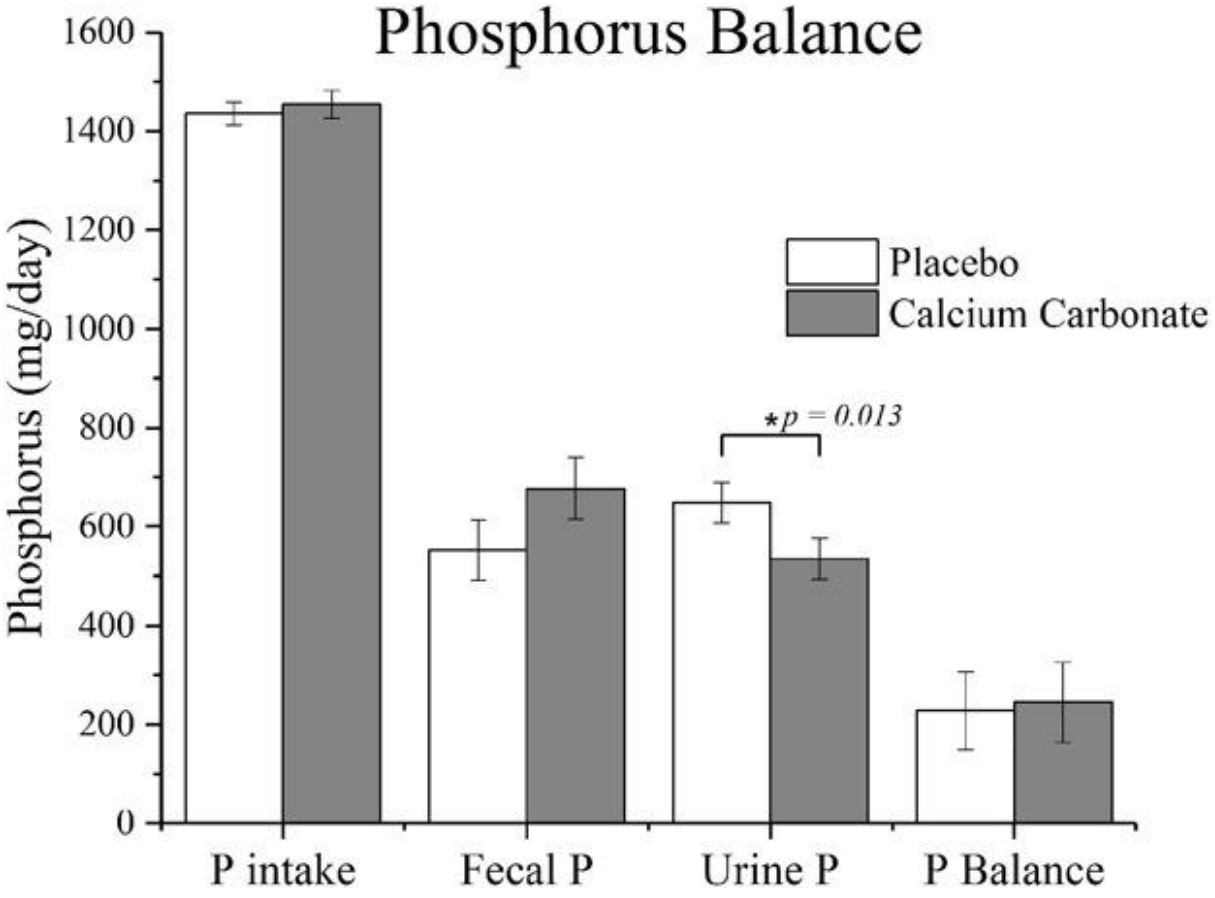

Figure 2.2. Phosphorus balance in healthy adolescent girls on placebo versus calcium carbonate.

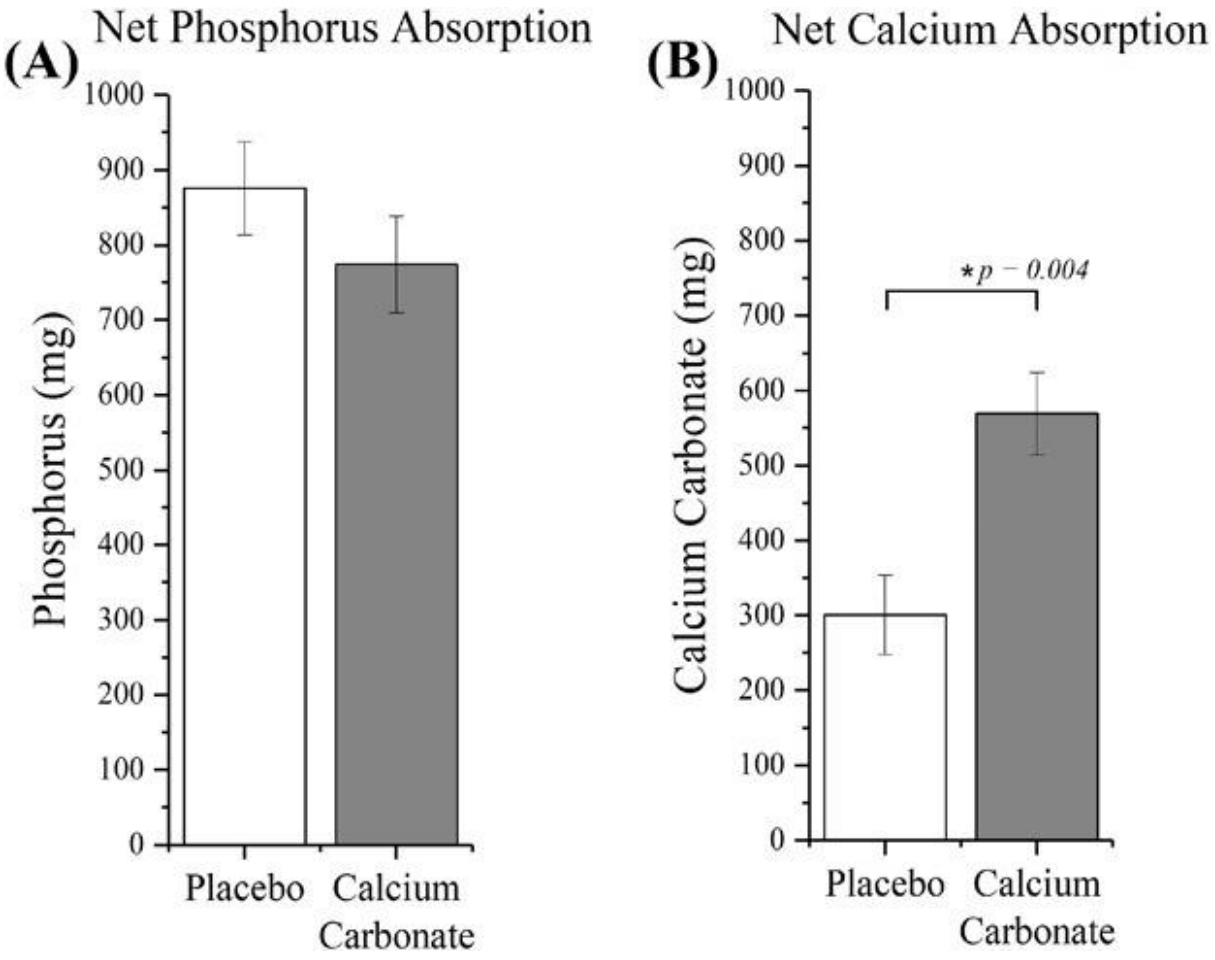

Figure 2.3. Net phosphorus and calcium absorption.
(*A*) Net phosphorus absorption in healthy adolescent girls on placebo versus calcium carbonate. (*B*) Net calcium absorption in healthy adolescent girls on placebo versus calcium carbonate ($n = 11$). Data from Elble and colleagues (1).

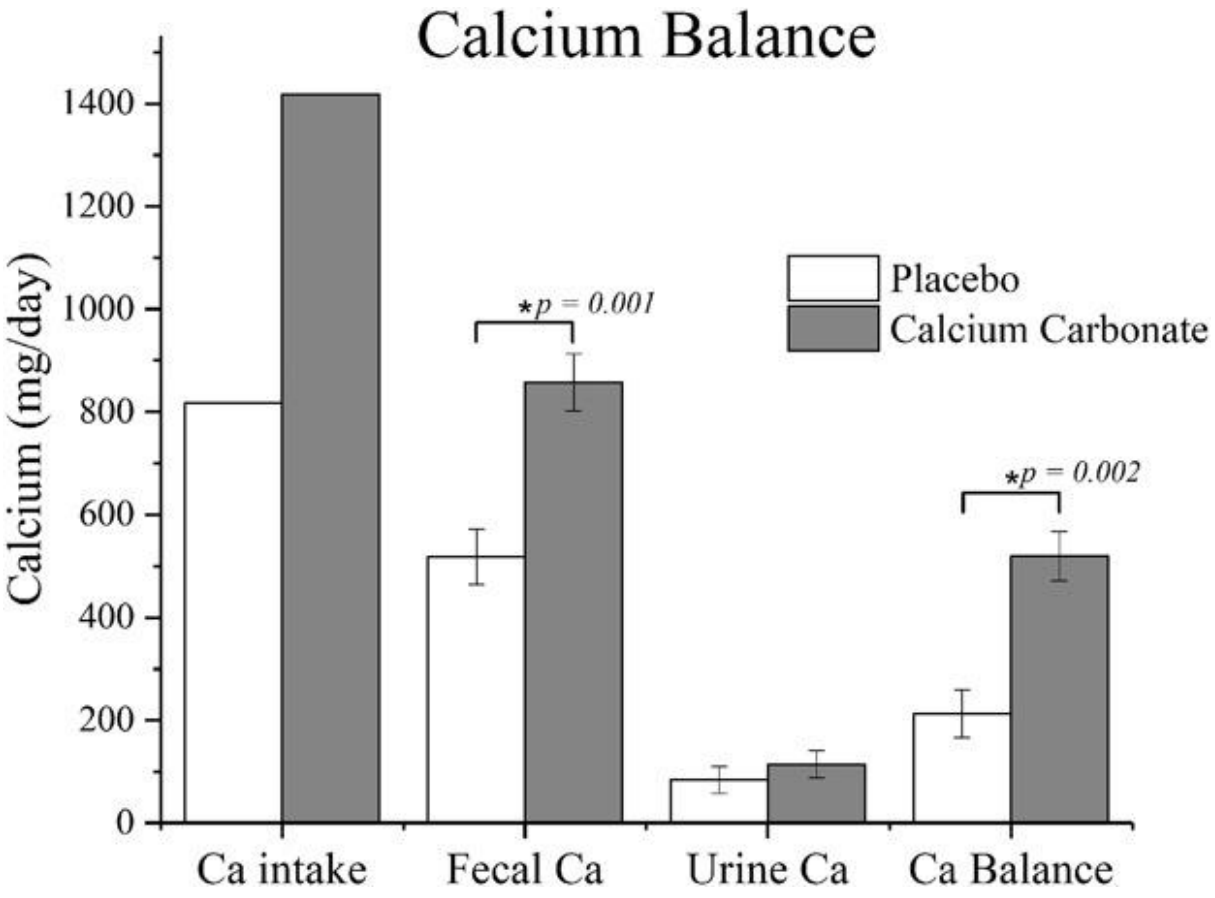

Figure 2.4. Calcium balance.
Calcium balance in healthy adolescent girls on placebo versus calcium carbonate ($n = 11$). Data from Elble and colleagues (1).

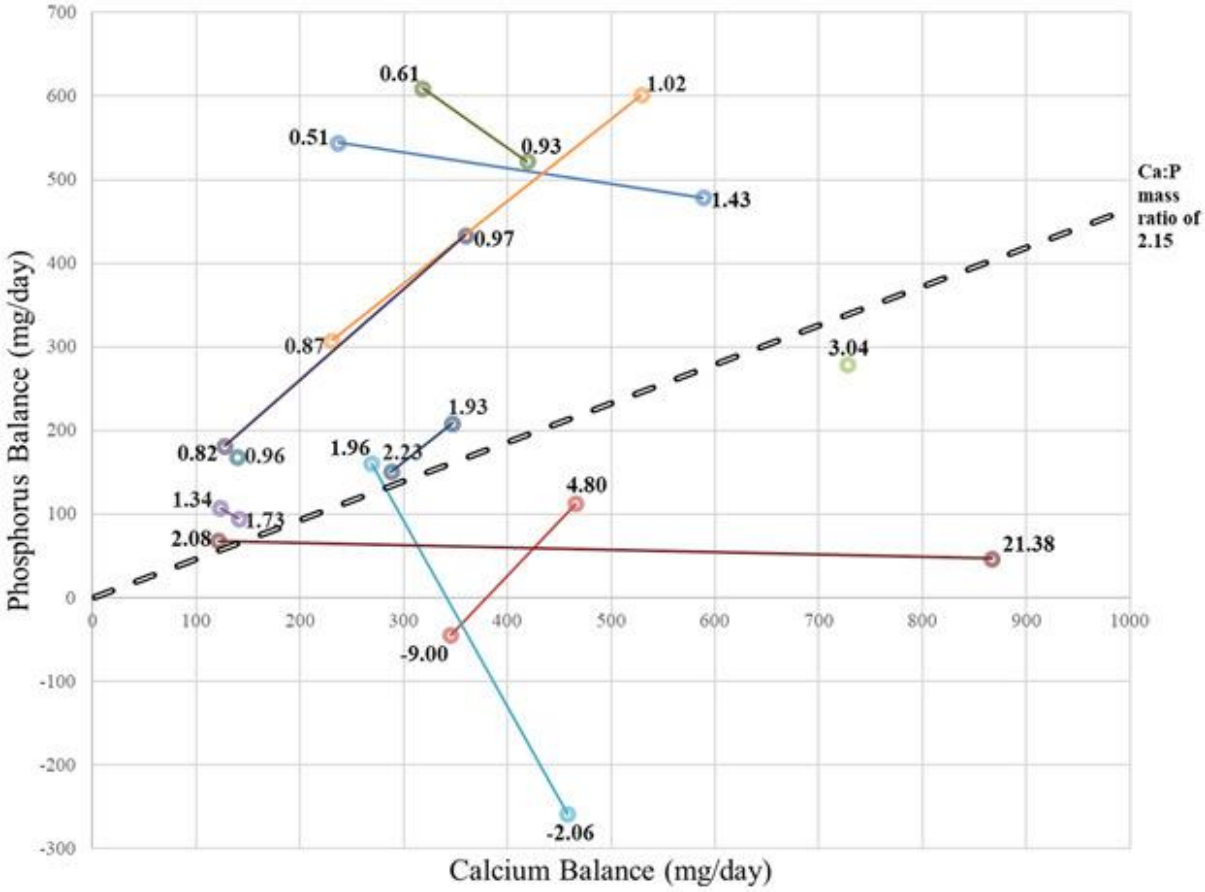

Figure 2.5. Phosphorus and calcium balance in individual participants on placebo and calcium supplement.
Ca:P mass ratio is given above each point, calculated from 99% Ca balance and 85% P balance. For comparison, the expected 2.15:1 Ca:P mass ratio of bone hydroxyapatite is shown by the dashed line.

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CHAPTER 3: EFFECT OF DIETARY PHOSPHORUS INTAKE AND AGE ON INTESTINAL PHOSPHORUS ABSORPTION EFFICIENCY AND PHOSPHORUS BALANCE IN MALE RATS

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Data available: https://doi.org/10.1371/journal.pone.0207601.s002

Abstract

Intestinal phosphorus absorption is an important component of whole-body phosphorus metabolism, and limiting dietary phosphorus absorption is particularly of interest as a therapeutic target in patients with chronic kidney disease to manage mineral bone disorders. Yet, mechanisms and regulation of intestinal phosphorus absorption have not been adequately studied and discrepancies in findings exist based on the absorption assessment technique used. *In vitro* techniques show rather consistent effects of dietary phosphorus intake level and age on intestinal sodium-dependent phosphate transport. But, the few studies that have used *in vivo* techniques conflict with these *in vitro* studies. Therefore, we aimed to investigate the effects of dietary phosphorus intake level on phosphorus absorption using the *in situ* ligated loop technique in three different aged rats. Male Sprague-Dawley rats (n = 72), were studied at 10-, 20-, and 30-weeks-of-age on a low (0.1%), normal (0.6%), or high (1.2%) phosphorus diet in a 3x3 factorial design (n = 8/group). Rats were fed their assigned diet for 2-weeks prior to absorption testing by jejunal ligated loop as a non-survival procedure, utilizing ^{33}P radioisotope. Metabolic cages were used for determination of calcium and phosphorus balance over the final four days prior to sacrifice, and blood was collected at the time of sacrifice for biochemistries. Our results show that phosphorus absorption was higher in 10-week-old rats compared with 20- and 30-week-olds and this corresponded to higher gene expression of the major phosphate

transporter, NaPi-2b, as well as higher whole-body phosphorus balance and net phosphorus absorption. Dietary phosphorus intake level did not affect jejunal phosphorus absorption or NaPi-2b gene expression. Our results contrast with studies utilizing *in vitro* techniques, but corroborate results of other rodent studies utilizing *in situ* or *in vivo* methods. Thus, there is need for additional studies that employ more physiological methods of phosphorus absorption assessment.

Introduction

Phosphorus is an essential nutrient for normal physiological function. However, elevated serum phosphorus has been linked to increased cardiovascular disease (1), bone disease (2), and mortality (3, 4). This is particularly true for patients with chronic kidney disease (CKD) (5), where the failing kidney has a reduced capacity for renal excretion. In normal physiology, the kidney is the primary site of regulation for phosphate homeostasis (6). Thus, as the kidneys fail, therapeutic options focus on reducing intestinal phosphorus absorption through dietary restriction, luminal phosphate binding, or inhibiting intestinal phosphorus transport. However, mechanisms and regulation of intestinal phosphorus absorption have not been adequately studied, especially when compared to that in the kidney. Renal phosphate reabsorption is nearly completely transcellular and sodium-dependent and is regulated by the major known phosphaturic hormones, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) (7). In contrast to the kidney, intestinal phosphorus absorption occurs by both sodium-dependent and sodium-independent pathways (8). However, the relative importance of each component is debated.

The majority of the existing literature on phosphorus absorption has relied on *in vitro* methods of absorption assessment including brush border vesicles or Ussing chambers. Low phosphorus diets have been shown to increase sodium-dependent intestinal phosphate uptake when measured by the rapid-filtration technique in isolated brush border membrane vesicles (BBMV) from healthy rats (9-12) and mice (13-16), and by Ussing chamber in rat (17) and pig (18). In contrast to in vitro studies, the limited in vivo studies in rodents have conflicting results with low phosphate diet both increasing (19) and having no effect on (20) intestinal phosphate absorption. Possible reasons for this discrepancy include the intestinal region tested (duodenum vs jejunum), sex and species of the animals (female Wistar vs male Sprague-Dawley), normal vs uremic animals, age of animals studied, differences in the technique used, and the duration of study. Given the importance of understanding precise mechanisms of intestinal phosphorus absorption *in vivo* in order to design more effective therapeutic interventions for patients with CKD, we studied rats at three ages with low, moderate, and high phosphorus diets. Since the majority of prior studies on intestinal phosphorus transport have utilized *in vitro* and *ex vivo* methods with results at odds with the limited *in situ/in vivo* results

available, we selected the *in situ* intestinal ligated loop method to assess phosphorus absorption by a more physiologic technique. Our findings show that intestinal phosphorus absorption by this method is affected by age, but is not affected by dietary phosphorus intake level, which conflicts with prior *in vitro* studies, but largely corroborates the limited prior *in situ/in vivo* studies.

Materials and Methods

Animals

This was a 3x3 factorial design study. Seventy-two male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were randomly assigned to 10-, 20-, or 30-week-old age groups, and randomly assigned to low, normal, or high dietary phosphorus within each age group (n = 8 rats/age x diet group). Rats were fed standard rat chow containing 0.7% phosphorus and 1.0% Ca (Harlan Teklad 2018, Indianapolis, IN) and water *ad libitum* until 8-, 18-, or 28-weeks of age (for the 10, 20, and 30-week-old age groups, respectively), at which time they were switched to their assigned study diets and fed *ad libitum* for two weeks prior to sacrifice. The low-phosphorus (LP), normal-phosphorus (NP), and high-phosphorus (HP) diets contained 0.1, 0.6, and 1.2% phosphorus, respectively, all with 0.6% Ca (Harlan Teklad, Indianapolis, IN: TD.85010, LP, TD.84122, NP, TD.85349, HP). Rats were housed individually in wire-bottom metabolic cages for the two weeks of the assigned diet period, and phosphorus and calcium balance were performed during the last four days prior to sacrifice. Body weights were taken weekly. The light-dark cycle was maintained from 6AM-6PM. This protocol was approved by the Purdue University Animal Care and Use Committee (Protocol Number: 1402001030).

Intestinal Phosphorus Absorption Efficiency

Intestinal phosphorus absorption efficiency was determined by *in situ* jejunal ligated loop absorption tests performed as a non-survival procedure before sacrifice. On day 14 of the assigned diet, rats were fasted for three hours prior to the ligated loop absorption test. Groups were order-balanced for treatment and testing to average the potential time-effect on absorption (21). The absorption test protocol was based on that published by Marks et al. (20), with the exception of the anesthetic. The rats were anesthetized by inhalation of isoflurane and kept warm with a heating blanket during the non-survival procedure for approximately 45 minutes. A

jugular vein catheter was placed for blood sampling, and a baseline blood sample (0.4 mL) was collected. The abdominal cavity was opened, and two ligatures were placed to create a ~5 cm segment of the jejunum. The first ligature was placed approximately 1 cm distal to the suspensory muscle of the duodenum (Ligament of Treitz) and firmly tied twice. The second ligature was loosely tied ~5 cm distal to the first ligature. Transport buffer (0.5 mL) containing (mmol/L) 16 Na-N-2-hydroxyethylpiperazine-N0-2-ethanesulfonicacid, 140 NaCl, 3.5 KCl, 0.1 KH2PO4, and ~5 uCi ^{33}P (^{33}P-orthophosphoric acid, PerkinElmer, Waltham, MA) was injected by gastight syringe (Hamilton, Reno, NV) into the jejunal lumen through the distal ligature, which was immediately tied off following the injection of the radioactive transport buffer. Blood (0.4 mL/sampling) was collected at 5, 10, 15, and 30 min post-injection in lithium heparin tubes and centrifuged at 10,500 g for 10 minutes (Micro 18R, VWR, Radnor, PA) to separate plasma. Immediately after the final 30-minute post-injection blood draw, the jejunal loop was removed, measured for length, and placed in a 20mL scintillation vial containing 6 mL Soluene-350 (PerkinElmer, Waltham, MA) for digestion in preparation for liquid scintillation counting of ^{33}P activity remaining in the jejunal loop. After heating overnight at 45°C in an oven, the dissolved jejunal loop was split into two vials and lightened with 0.6 mL 30% hydrogen peroxide (Avantor, Center Valley, PA) to reduce color quench.

Under anesthesia, rats were sacrificed by cardiac puncture and exsanguination followed by cardiac excision. Blood (0.5 mL) was aliquoted into lithium heparin tubes for hematocrit measurement and stored on ice until centrifugation for 5 minutes at 5,900 g (Readacrit, Clay Adams, Parsippany, N.J.). Remaining blood was aliquoted into lithium heparin tubes for plasma separation as described above, flash frozen in liquid nitrogen and stored at -80C until analysis.

Liquid scintillation counting of the ^{33}P transport buffer solution, plasma samples from each time point, and digested jejunal loop was performed on a Tri-Carb 2910 TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA). 500 uL of transport buffer solution and 250 uL plasma samples were counted in 15 mL of EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA), and digested loops were counted in 17 mL of Hionic-Fluor scintillation cocktail (PerkinElmer, Waltham, MA). Appropriate quench curves for each scintillation cocktail were used to adjust counts per minute to disintegrations per minute (22) (Supporting Information). Absorption of ^{33}P was evaluated two ways: 1) area under the curve

(AUC) was calculated for plasma ^{33}P activity over 30 minutes, and 2) percent intestinal phosphorus absorption efficiency over 30 minutes was calculated as:

$1 - (^{33}P \text{ activity remaining in digested loop}) / (\text{Total } ^{33}P \text{ activity in 0.5mL dose}) \cdot 100$

Phosphorus and Calcium Balance and Net Absorption

On days 10 through 14 of the assigned study diet, all urine and feces were collected and diet weighed daily to assess 4-day average phosphorus and calcium balance and net absorption. Feces and diet were ashed in a muffle furnace (Thermolyne Sybron Type 30400, Dubuque, IA) for 10 days at 550°C. Feces were then diluted 140X and diet 14X with 2% nitric acid. Urine was diluted 11X with 2% nitric acid. Phosphorus and calcium in urine, feces, and diet were quantified by inductively coupled plasma-optical emission spectrophotometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT). Urine creatinine was determined by colorimetric method using a COBAS Integra 400 Plus (Roche Diagnostics, Indianapolis, IN). Four-day phosphorus balance was calculated as dietary phosphorus intake (mg/d) minus urine and fecal phosphorus excretion (mg/d), and net phosphorus absorption as phosphorus intake (mg/d) minus fecal excretion (mg/d). Calcium balance and net calcium absorption were calculated similarly.

Intestinal Phosphate Transporter Gene Expression

After the completion of the ligated loop absorption tests and the removal of the radioactive jejunal loop, approximately 5 cm of jejunum distal to the loop and 5 cm of duodenum distal to the pylorus were removed, cut open and rinsed with ice-cold deionized water. The mucosal layers were scraped, and mucosa from each intestinal segment was placed into TRI Reagent (Fisher Scientific, Hampton, NH) and flash frozen in liquid nitrogen for later mRNA quantification by RT-PCR.

Total RNA was extracted using Omega HP Total RNA Kit (R6812-00, Omega Bio-tek, Norcross, GA; modified to add a chloroform extraction step). Concentration and purity were determined on a NanoDrop 2000c spectrophotometer at 260, 280, and 230nm (Thermo Fisher Scientific, Waltham, MA). Real-time PCR amplifications were performed using TaqMan gene expression assays (TaqMan MGP probes, FAM dye-labeling) with Applied Biosystems ViiA 7 Real-Time PCR systems (Applied Biosystems). NaPi2b (Slc34a2), PiT1 (Slc20a1), and Rplp0

primers were obtained from Applied Biosystems (Rn00584515_m1, Rn00579811_m1, and Rn03302271_gH). The ΔΔCT method was used to analyze the relative change in gene expression normalized to the housekeeping gene Rplp0.

Plasma Biochemistries

Plasma stored at -80C was thawed and analyzed for phosphorus, calcium, and creatinine concentration by colorimetric method using a COBAS Integra 400 Plus (Roche Diagnostics, Indianapolis, IN). Blood urea nitrogen (BUN), calcium and phosphorus were measured by colorimetric assay (BioAssay Systems, Hayward, CA and Point Scientific, Canton, MI). Intact parathyroid hormone (iPTH), intact fibroblast growth factor 23 (iFGF23), c-terminal (includes c-terminal fragments and intact protein) FGF23 (cFGF23) (Immutopics, San Clemente, CA), and 1,25(OH)2D3 by enzyme immunosorbent assay (Immunodiagnostic Systems, The Boldons, UK).

Statistics

A sample size of n = 8 rats/group was determined to be sufficient to detect a 30% difference between groups for phosphorus absorption ($\beta = 0.80$, $\alpha = 0.05$) based on means and standard deviations reported by Marks et al. (20). Two-way ANOVA was performed for all outcomes with main effects for diet and age and their interaction utilizing least-squares means with Tukey post-hoc comparisons. Statistical significance was set at $\alpha < 0.05$. Statistical Analysis Software version 9.3 (SAS Institute, Cary, NC) was used for all statistical analysis. Results and figures are reported as mean ± SEM of each age group on the three diets, or vice versa, unless otherwise indicated.

Results

Body weight prior to starting the assigned diet was higher for older rats, as physiologically expected (10-week-olds: 275.8 ± 3.1 g, 20-week-olds: 425.5 ± 5.5 g, 30-week-olds: 461.5 ± 5.5 g, $p < 0.0001$ for all comparisons, but was not different between the diet groups, nor was there an age x diet interaction (diet main effect, $p = 0.3751$; age x diet interaction, $p = 0.2762$). At sacrifice, body weight followed a similar pattern (10-week-olds:

306.7 ± 5.8 g, 20-week-olds: 438.7 ± 4.5 g, 30-week-olds: 485.8 ± 5.7 g, $p < 0.0001$ for all comparisons; diet main effect, $p = 0.1344$; age x diet interaction, $p = 0.0741$).

At the time of sacrifice, plasma creatinine tended to be higher in 10-week-old rats versus 20- and 30-week-olds (0.49 ± 0.02 mg/dL vs 0.44 ± 0.01 mg/dL and 0.45 ± 0.01 mg/dL) but no post-hoc comparisons were significant due to a marginal interaction with diet (Table 3.1). Urinary creatinine increased at each age group (7.2 ± 0.2, 11.9 ± 0.4, 13.5 ± 0.4 for 10, 20, and 30-week-old, respectively ($p < 0.0001$ for 10 vs 20 and 30, $p = 0.0042$ for 20 vs 30). (Table 3.1). Creatinine clearance was lower in 10-week-old rats versus 20- and 30-week-old rats (3.6 ± 0.1 mL/min vs 4.5 ± 0.2 mL/min and 4.5 ± 0.1 mL/min, $p = 0.0005$ and $p = 0.0003$, respectively) (Table 3.1). Plasma BUN progressively declined with age but was within normal physiologic range (22.6 ± 0.5 mg/dL, 20.4 ± 0.6 mg/dL, and 18.3 ± 0.5 mg/dL for 10-, 20-, and 30-week groups respectively ($p < 0.015$ for all comparisons) and did not change with level of phosphorus in the diet ($p = 0.0771$). Plasma phosphorus was higher in 10-week old rats compared to both the 20- and 30-week olds (9.4 ± 0.3 mg/dL vs 7.5 mg/dL ± 0.2 and 7.2 ± 0.1 mg/dL, $p < 0.0001$ for both), but there was no difference between diets (Table 3.1). There was a significant age x diet interaction for plasma calcium, where 10-week olds had higher plasma calcium on LP compared with NP and HP ($p = 0.0011$ and $p = 0.0005$ respectively), but there were no other significant group comparisons (Table 3.1). Overall, dietary phosphorus level and age caused anticipated changes in the phosphorus-regulating hormones of 1,25D, FGF23, and PTH. There was a significant age x diet interaction for plasma iPTH where LP resulted in lower iPTH compared with NP and HP, but the magnitude was greatest in the 10-week old rats (Table 3.1). Both iFGF23 and cFGF23 were lower in the LP vs NP and HP (iFGF23: 106.6 ± 11.3 pg/mL vs 327.1 ± 14.7 pg/mL and 347.3 ± 11.8 pg/mL, $p < 0.0001$ for both; cFGF23: 266.6 ± 15.1 pg/mL ± vs 496.8 ± 16.6 pg/mL and 514.8 ± 21.4 pg/mL, $p < 0.0001$ for both) (Table 3.1). iFGF23 was also lower in 10-week-old vs 20-week-old rats (240.7 ± 27.7 pg/mL vs 282.5 ± 25.6 pg/mL, $p = 0.0491$) (Table 3.1). 1,25D was higher in 10-week old rats compared to 20- and 30-week (446.0 ± 19.9 pg/mL vs 339.9 ± 16.7 pg/mL and 325.1 ± 16.6 pg/mL, $p = 0.0001$ and $p < 0.0001$) and it was higher on LP vs NP (414.1 ± 24.7 pg/mL vs 328.7 ± 18.0 pg/mL; $p = 0.0017$), HP was not significantly different from LP (370.1 ± 16.9 pg/mL vs 414.1 ± 24.7 pg/mL, $p = 0.11$) or NP (370.1 ± 18.0 pg/mL vs 328.7 pg/mL, $p = 0.24$) (Table 3.1).

Percent intestinal phosphorus absorption efficiency (percent of dose), as assessed by disappearance from the intestinal loop at 30 minutes, was higher in 10-week-olds compared to both 20- and 30-week-olds (42.5 ± 0.02% vs 35.6 ± 0.01% and 34.7 0.02%, $p < 0.01$ for both), but 20- and 30-week-olds were similar (Fig 3.1). Correspondingly, the plasma ^{33}P activity (percent of dose) AUC over 30 minutes was higher in 10 weeks-olds compared to both 20- and 30- week-olds (1.3 ± 0.08% vs 0.7 ± 0.03% and 0.7 ± 0.04%, $p < 0.0001$ for both) (Fig 3.2) There was no effect of dietary phosphorus level on either absorption measure (loop: $p = 0.4907$; plasma AUC: $p = 0.2585$), nor any significant age x diet interaction (loop: $p = 0.4034$; plasma AUC: $p = 0.9986$). Similar results were observed when plasma ^{33}P activity at only the final 30-minute time point was analyzed ($p = 0.6742$ for diet main effect, $p < 0.0001$ for 10 week-olds vs 20- and 30-week-olds).

Similar to the jejunal ligated loop absorption results, jejunal NaPi-2b mRNA was higher in 10-week-olds compared to both 20- and 30-week-olds ($p = 0.0016$ and $p = 0.0245$, respectively) (Fig 3.3A). In the duodenum, there was an age x diet interaction ($p = 0.0011$) driven by higher NaPi-2b mRNA levels in 10-week-olds on the low phosphorus diet compared to all other groups (Fig 3.3B). Jejunal PiT1 mRNA tended to be higher at 30-weeks, although the overall model did not reach statistical significance ($p = 0.0524$) (Fig 3.3C). Duodenal PiT1 expression was not significantly affected by diet or age (overall model $p = 0.4975$) (Fig 3.3D).

Phosphorus balance was higher in 10-week-olds compared with both 20- and 30-week-olds, and not different between 20- and 30-week-olds (36.4 ± 5.5 mg/day vs 15.7 ± 6.1 mg/day and 18.1 ± 5.5 mg/day, $p = 0.0113$ and 0.0286, respectively) (Fig 3.4A). There was a significant effect for diet on phosphorus balance, where HP was higher than NP and LP (43.4 ± 8.2 mg/day vs 17.5 ± 3.4 mg/day and 9.2 ± 1.2 mg/day, $p = 0.0012$ and $p < 0.0001$, respectively). For net phosphorus absorption, there was a clear dose-response effect of diet (LP: 9.4 ± 1.2 mg/day, NP: 62.0 ± 2.7 mg/day, HP: 162.2 ± 3.8 mg/day, $p < 0.0001$ for all comparisons), and 10-week-olds had higher net phosphorus absorption than 20- and 30-week old rats (85.3 ± 13.5 mg/day vs 76.4 ± 13.6 mg/day and 72.0 ± 13.3 mg/day, $p = 0.0469$ and $p < 0.0017$, respectively), but 20- and 30-week-olds were not different (Fig 3.5A)**.** Calcium balance was also higher in 10-week-old rats compared with both 20- and 30-week but not between 20- and 30-week-olds (32.5 ± 1.7 mg/day vs 15.8 ± 2.9 mg/day and 7.8 ± 4.6 mg/day, $p = 0.0024$ and $p < 0.0001$, respectively (Fig 3.4B). Net calcium absorption was similarly higher in 10-week rats (35.7 ± 2.1 mg/day vs 17.5 ± 3.0

mg/day and 10.1 ± 4.7 mg/day, $p = 0.0012$ and $p < 0.0001$) (Fig 3.5B). Phosphorus in the diet had no effect on calcium balance nor net absorption of calcium ($p = 0.6981$ and $p = 0.1141$ for main effect) (Figs 3.4B and 3.5B).

Discussion

In this study, we found higher intestinal phosphorus absorption at 10-weeks of age compared to 20- and 30-weeks as assessed by the ligated loop technique in both appearance of ^{33}P in plasma and disappearance of ^{33}P from the intestinal loop. This interpretation is supported by the more positive net phosphorus absorption from metabolic balance and more positive overall phosphorus balance, higher plasma phosphorus, and higher NaPi-2b mRNA expression in the 10-week rats vs the 20- and 30-week-olds. The lack of differences between 20- and 30-week-old rats is likely due to less metabolic demand of bone for phosphorus. The increased phosphorus absorption and positive phosphorus balance corresponded to higher serum phosphorus levels at a younger age, similar to that in humans (23, 24). Further, there was lower PTH and FGF23 levels and higher 1,25D levels at the younger age, suggesting that hormonal regulation decreases renal phosphorus excretion via decreased PTH and FGF23, and increases intestinal absorption via vitamin D (7). The elevation at 10-weeks likely reflects the increased requirement of phosphorus for growth at this age (24) but how these hormonal changes are stimulated during growth is not completely understood.

Other studies that have observed differences in sodium-dependent BBMV phosphate uptake between post-weaning and adult rodents have studied even older rats and still found no differences compared with younger adult rats. Armbrecht (25) compared 8–12 week-old (“young’) vs 48-46-week-old (“adult”) and 88-96-week-old (“old”) Fisher 344 rats, and observed differences between the young vs adult and old rats, but no difference between the adult and old rats. This suggests that sodium-dependent absorption decreases after growth then plateaus for the adult lifespan. Borowitz and Ghishan (26) also showed an age-dependent decrease in jejunal sodium-independent BBMV phosphate uptake from 2 to 6 weeks-of-age, albeit smaller than sodium-dependent reductions. The changes to sodium-independent phosphate transport in later stages of aging should be further evaluated, as this may contribute to a greater portion of total phosphorus absorption with aging. In the pre-weaning mouse, an age-dependent decrease in

NaPi-2b occurs from 14 days to 21 days to 8 weeks, and then remains the same until 8–9 months (32–36 weeks) (27). In rats, there is an age-dependent decrease in NaPi-2b gene expression from 2 weeks to 3 weeks to 6 weeks and 95–100 days (13.5–14.3 weeks), and a reduction in BBMV uptake between 2 weeks and 95–100 days (28). Future work is necessary to characterize changes in absorption during this transition using more physiologic absorption techniques.

Importantly, we did not find a difference by dietary phosphorus level on intestinal phosphorus absorption assessed by jejunal ligated loop. We used the same low phosphorus diet (0.1%) that has been shown to increase jejunal NaPi-2b expression and sodium-dependent phosphate uptake in rats *in vitro* (11, 12), but don't see that translate in our study to an effect on the *in situ* ligated loop technique using a transport buffer phosphate concentration that would be expected to favor sodium-dependent transport (29). The sodium-dependent, transcellular pathway predominates at low luminal phosphate concentrations, whereas the sodium-independent, paracellular pathway will contribute more at high luminal phosphate concentrations (29-31). The latter was reflected in our results by the stepwise increase in net phosphorus absorption corresponding to the amount of phosphorus in the diet. NaPi-2b is currently understood to be the main sodium-dependent intestinal phosphate transporter. It shares homology to the renal NaPi-2a/c transporters (32, 33) and the type III sodium-phosphate co-transporters, PiT-1 and PiT-2, that are considered to play more minor roles in absorption (32-36). NaPi-2b is estimated to contribute >90% of sodium-dependent transport based on a mouse NaPi-2b knockout model (35), with PiT-1 and PiT-2 believed to contribute <10% of sodium-dependent transport (36). We did not measure PiT-2 because its expression in the intestine is very low (11) and others have found that PiT-2 mRNA does not change in response to dietary phosphorus restriction (10). Interestingly, recent evidence suggests that additional transporters may be yet undiscovered (12). Our data question the importance of sodium-dependent transcellular phosphate transport in the presence of a liberal consumption of dietary phosphorus which would favor the sodium-independent pathway, characteristic of the American diet (37). Other rodent and pig studies have consistently found increases in *in vitro* intestinal BBMV phosphate uptake after a low phosphorus diet (9-17), and while studies that measure NaPi-2b protein expression consistently find increases (11, 12, 14-16, 18, 38), NaPi-2b mRNA expression only increases in some (14, 16, 38), but not all studies (10, 15, 20). A limitation in our study is a lack of transporter protein expression. In contrast, and similar to our findings, the previous studies in

rodents using the more physiologic *in situ* ligated loop technique for effects of low phosphorus diets have had conflicting results (19, 20). Rizzoli et al. (19) observed higher duodenal phosphate transport after a 15-minute ligated loop on a low phosphorus (0.2%) diet in normal female rats compared to a normal phosphorus (0.8%) diet after 16 days of feeding, but not after only 8 days, and higher duodenal phosphate transport with a normal phosphorus (0.8%) diet compared to a high phosphorus (1.8%) diet after 8 days. However, these results were split between two separate experiments with different concentrations of phosphate in the absorption buffers (5 mM and 2 mM), making it difficult to compare low vs high phosphorus intake. More recently, Marks et al. (20) tested the effects of very low phosphorus (0.02%) versus normal phosphorus (0.52%) diet on jejunal phosphate transport efficiency in 5/6th nephrectomized male rats with a 30-minute jejunal ligated loop. No effect of diet on phosphorus absorption was observed, nor any change in jejunal or duodenal NaPi-2b mRNA expression. Together, the results of our study which utilized both loop disappearance and plasma ^{33}P counts, and the other *in situ* studies, suggest that conclusions on the effects of low phosphorus diets on phosphorus absorption from studies utilizing *in vitro* and *ex vivo* uptake/transport or flux techniques which utilize isolated intestinal segments or BBMV may not appropriately reflect physiologic conditions affecting phosphorus absorption. Thus, there is need for additional studies that employ more physiological methods of phosphorus absorption assessment. Our study was limited to male rats, so we were unable to determine if sex-differences exist for age and dietary phosphorus intake level effects on intestinal phosphorus absorption by the ligated loop technique. To our knowledge, ours is the first study to utilize the ligated loop method on normal male rats to test this question.

In vitro techniques may fail to replicate physiologic *in vivo* techniques for a number of reasons. First, even when using a low transport buffer phosphate concentrations (0.1 mM KH_2PO_4) (30) close to the Km of NaPi-2b (39), residual luminal phosphate makes it such that sodium-dependent transport is contributing a smaller proportion to total phosphate transport and mask effects of interventions on the sodium-dependent transport mechanism. Luminal phosphate concentration *in vivo* is ~1.5–40 mM in the proximal intestine depending on measurement technique (30, 40), which may contribute to a higher passive transport than *in vitro* techniques. Thus, estimating this contribution with the ligated loop is challenging. Marks and colleagues assessed the proportion of sodium-dependent absorption with the ligated loop in rats at 32 ± 8% vs 73 ± 5% using the everted sleeve with the phosphate concentration that we utilized (30). It is

likely that excreted luminal sodium during the test results in sodium-dependent transport when injecting a solution without sodium, and indeed measurable sodium was present at the end of the test. Therefore, we assume that 32% sodium-dependency is a low-end estimate using this technique. While it is a limitation that we didn't attempt to measure changes in sodium-independent absorption with respect to dietary phosphorus and age, sodium-independent uptake is not regulated by dietary phosphorus (10, 13, 15, 41). Additional factors such as a disconnection to local blood flow and nerves or transmural potential differences may explain *in situ*/*in vivo* differences with *in vitro/ex vivo* techniques (30). It is also possible that other intestinal segments would respond to the two factors we tested. However our interest in studying the jejunum as regional specific regulation of phosphorus absorption was because 1) NaPi-2b is expressed highest in the jejunum in rats (42), 2) some studies show that NaPi-2b mRNA and protein only respond to chronic phosphorus restriction in the jejunum but not duodenum (11), and only uptake increased in the jejunum (11). Further, infusion of Matrix Extracellular Phosphoglycoprotein in rats led to a selective change in absorption rate in the jejunum but not duodenum, suggesting that the jejunum may be most responsive to other factors as well (43). However, Rizzoli and colleagues showed an increased absorption efficiency in the duodenal segment in rats with the loop (19), whereas others have showed age related decreases in uptake with age in both the jejunum and duodenum (25). Additional research is needed to clarify the relative importance of capacity of the duodenum to adapt to various factors.

In conclusion, in the present study we examined the interaction of age and diet on intestinal phosphorus absorption in healthy male Sprague Dawley rats with the *in situ* ligated loop technique. Moderate phosphorus restriction (0.1%) did not affect phosphate absorption with this technique, but absorption was higher in younger (10-week) rats with higher positive phosphorus balance.

Table 3.1. Final blood and urine biochemistries†.

	10 weeks old			20 weeks old			30 weeks old			P-Values			
	LP	NP	HP	LP	NP	HP	LP	NP	HP	Model	Age	Diet	Age x Diet
Plasma P (mg/dL)[a]	9.2 (0.6)	9.0 (0.2)	9.9 (0.5)	7.1 (0.3)	8.0 (0.2)	7.4 (0.3)	7.2 (0.3)	7.2 (0.2)	7.2 (0.2)	**<0.0001**	**< 0.0001**	0.4715	0.1703
Plasma Ca (mg/dL)[a]	10.8 (0.4)	9.4 (0.1)	9.3 (0.1)	9.6 (0.2)	9.1 (0.2)	9.3 (0.2)	9.6 (0.2)	9.7 (0.1)	9.8 (0.1)	**<0.0001**	**0.0084**	**0.0004**	**0.0009**
Plasma Creatinine (mg/dL)[a]	0.5 (0.04)	0.5 (0.02)	0.5 (0.04)	0.5 (0.02)	0.5 (0.02)	0.4 (0.02)	0.5 (0.01)	0.5 (0.02)	0.4 (0.0)	**0.0142**	**0.0438**	0.1500	0.0504
Urine Creatinine (mg/dL)	6.6 (0.4)	8.0 (0.5)	7.2 (0.1)	12.4 (0.8)	10.7 (0.7)	12.5 (0.7)	13.3 (0.7)	14.3 (0.4)	12.8 (0.7)	**<0.0001**	**< 0.0001**	0.8948	0.0317
Hematocrit (%)[b]	42.2 (1.1)	43.1 (0.7)	44.3 (0.5)	44.6 (1.0)	45.3 (0.7)	44.1 (1.4)	44.3 (0.1)	43.9 (1.0)	45.5 (0.7)	0.2573	0.0828	0.4396	0.4635
Creatinine Clearance (mL/min)[a]	3.2 (0.2)	4.0 (0.2)	3.5 (0.2)	4.5 (0.4)	4.0 (0.3)	5.0 (0.3)	4.3 (0.3)	4.2 (0.2)	4.9 (0.2)	**0.0002**	**<0.0001**	0.0814	0.0641
BUN (mg/dL)[c]	23.6 (0.9)	21.8 (0.8)	22.5 (0.7)	22.0 (0.8)	21.0 (0.9)	18.4 (1.2)	18.7 (0.7)	18.0 (0.9)	18.3 (1.3)	**<0.0001**	**<0.0001**	0.0771	0.2731
Plasma PTH (pg/mL)	92.1 (28.9)	617.1 (43.7)	778.6 (64.5)	194.6 (37.9)	446.7 (40.6)	376.6 (54.9)	262.8 (62.2)	437.2 (95.6)	421.6 (49.0)	**<0.0001**	**0.0027**	**<0.0001**	**<0.0001**
Plasma iFGF23 (pg/mL)[d]	60.2 (4.0)	300.9 (19.1)	338.3 (24.9)	133.0 (21.1)	340.0 (32.9)	355.8 (21.0)	124.2 (16.1)	340.4 (23.5)	347.8 (16.8)	**<0.0001**	**0.0377**	**<0.0001**	0.7148
Plasma cFGF23 (pg/mL)[d]	292.3 (34.0)	504.0 (19.2)	554.4 (48.6)	240.8 (13.3)	458.5 (37.2)	497.6 (39.8)	266.6 (21.6)	528.0 (24.9)	492.3 (13.1)	**<0.0001**	0.1426	**<0.0001**	0.6751
Plasma 1,25D (pg/mL)[e]	527.7 (30.1)	404.0 (25.7)	411.3 (29.5)	385.2 (25.3)	289.0 (32.0)	356.7 (17.7)	336.3 (31.4)	302.5 (19.7)	338.2 (36.5)	**<0.0001**	**<0.0001**	**0.0026**	0.2225

Table 3.1 cont.

†**Final blood and urine biochemistries**. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown, and means and (SEM) are shown for each group. Plasma phosphorus values were higher in 10 week olds vs 20 and 30 weeks. An age x diet interaction for plasma calcium was driven by an increase at 10 weeks on the low phosphorus diet. Plasma creatinine was higher in 10 week olds but had a marginal interaction with diet. Urinary creatinine increased at each age group. Blood hematocrit was not different between age or diet groups. Creatinine clearance was lower at 10 weeks vs 20 and 30 weeks. Plasma BUN progressively declined with age. There was a significant age x diet interaction for plasma PTH were low phosphorus resulted in lower PTH compared to normal and high phosphorus, but the magnitude was greatest at 10 weeks. iFGF23 was lower in the low phosphorus group compared to normal and high, and lower at 10 weeks compared to 20 weeks. cFGF23 was lower in the low phosphorus group compared to normal and high phosphorus. Plasma 1,25D was higher at 10 weeks compared to 20 and 30 weeks, and higher on the low phosphorus compared to normal phosphorus diet. LP = low phosphorus diet, NP = normal phosphorus diet, HP = high phosphorus diet.
[a] n = 4 excluded for insufficient plasma. [b] n = 12 excluded for insufficient sample. [c] n = 2 excluded for insufficient plasma. [d] n = 3 excluded as unphysiologic outliers (near zero). [e] n = 6 excluded for insufficient plasma.

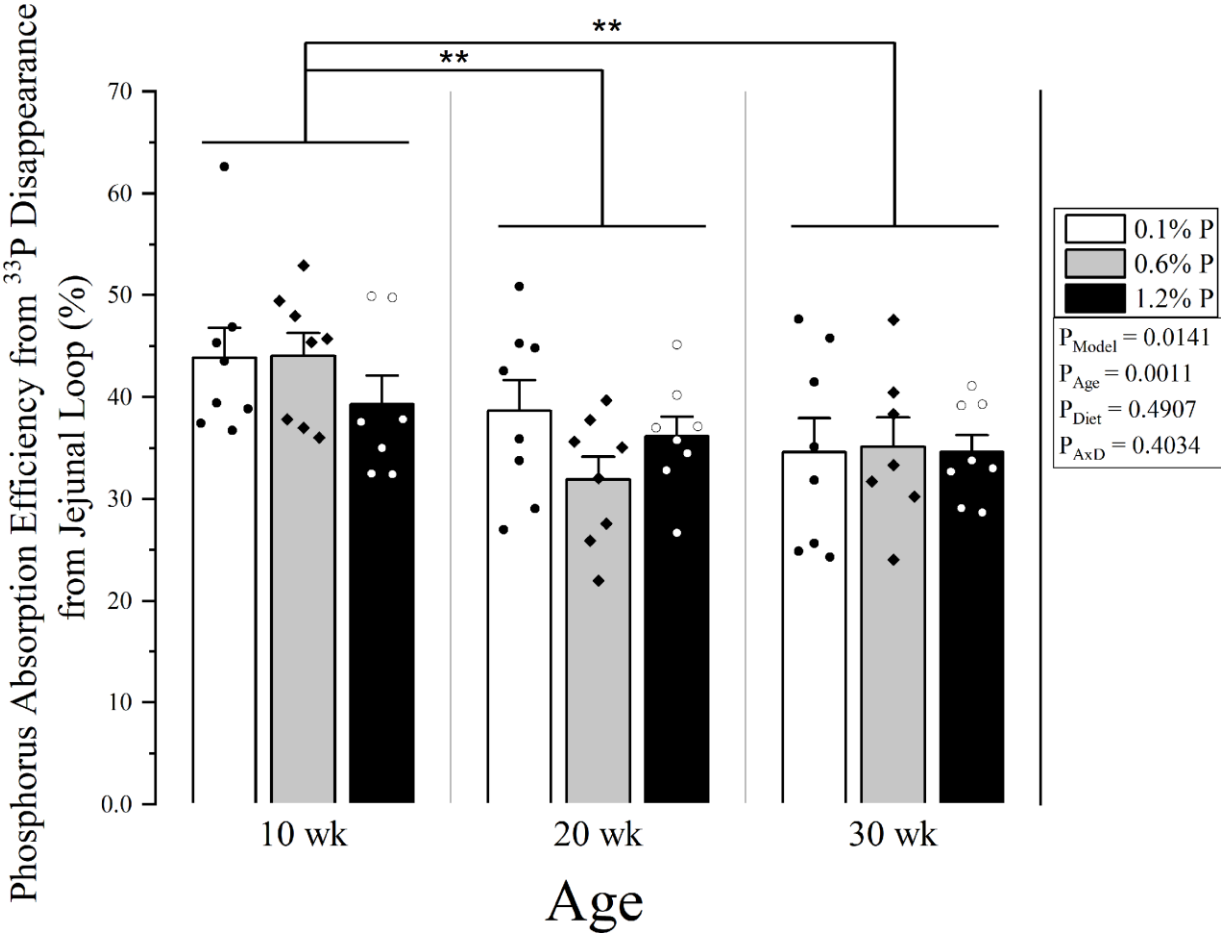


Figure 3.1. Percent jejunal phosphorus absorption efficiency by age and dietary phosphorus intake level.

Phosphorus absorption efficiency was calculated as 1-(^{33}P activity remaining in jejunal loop)/(Total ^{33}P activity in dose) after 30 minutes post ^{33}P injection into the jejunal loop. Means and standard error bars are shown for each group. Low phosphorus diet (0.1%) is shown in white bars and black dots; normal phosphorus diet (0.6%) is shown in grey with black diamonds; and high phosphorus diet (1.2%) is shown in black with white circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown. There was a main effect for age where 10 week old rats had higher phosphorus absorption compared to both 20 and 30 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. ** $p < 0.01$. n = 2 excluded, 1 for mishandling loop, and 1 unphysiologic (counts ~150 fold lower than expected).

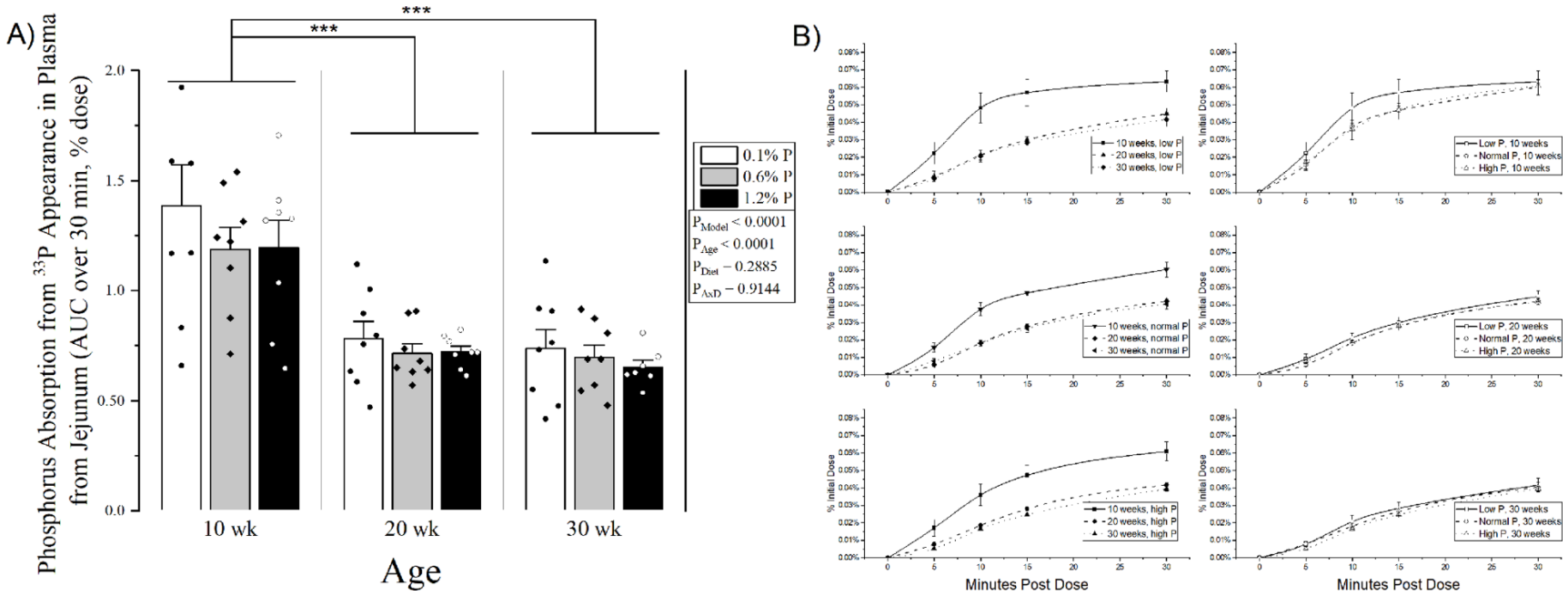


Figure 3.2. Jejunal phosphorus absorption into plasma.

A) Jejunal phosphorus absorption determined by appearance of ^{33}P in plasma over 30 minutes (AUC). Means and standard error bars are shown for each group. Low phosphorus diet (0.1%) is shown in white bars and black dots; normal phosphorus diet (0.6%) is shown in grey with black diamonds; and high phosphorus diet (1.2%) is shown in black with white circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown. B) Jejunal phosphorus absorption determined by appearance of ^{33}P in plasma over 30 minutes (time series). Each diet group (left) and each age group are compared (right). Results at 30 minutes were similar to AUC. Absorption was calculated as percent of total ^{33}P activity in the initial dose. There was a main effect for age where 10 week old rats had higher phosphorus absorption compared to both 20 and 30 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. *** $p < 0.0001$. n = 1 excluded for missing last 2 timepoints.

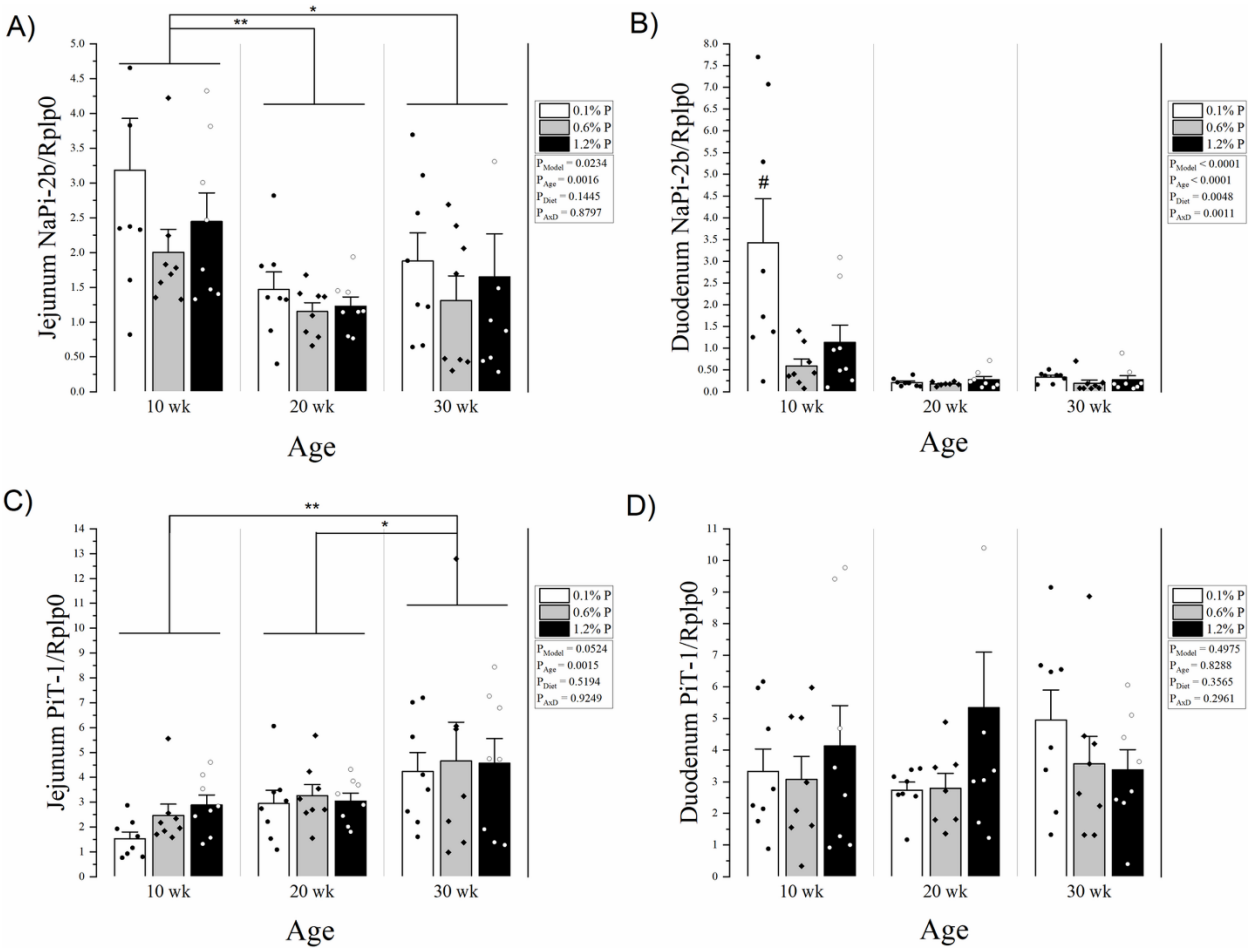


Figure 3.3. Intestinal phosphate transporter gene expression.
A) Jejunal NaPi-2b mRNA expression by age and dietary phosphorus intake level.There was a main effect for age where 10 week old rats had higher NaPi-2b expression compared to both 20 and 30 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. B) Duodenal NaPi-2b mRNA expression by age and dietary phosphorus intake level. There was a significant interaction driven by a higher expression in 10 week rats on a low phosphorus diet. C) Jejunal PiT-1 mRNA expression by age and dietary phosphorus intake level. The overall model approached significance, with a main effect for age where 30 week old rats had higher PiT-1 expression compared to both 20 and 10 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. D) Duodenal PiT-1 mRNA expression by age and dietary phosphorus intake level. There were no differences in age, diet, or interaction. Expression was calculated relative to Rplp0. Means and standard error bars are shown for each group. Low phosphorus diet (0.1%) is shown in white bars and black dots; normal phosphorus diet (0.6%) is shown in grey with black diamonds; and high phosphorus diet (1.2%) is shown in black with white circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown. * $p < 0.05$, ** $p < 0.01$, # $p < 0.01$ vs all other groups. n = 1 duodenum NaPi-2b and PiT-1 excluded for missing sample.

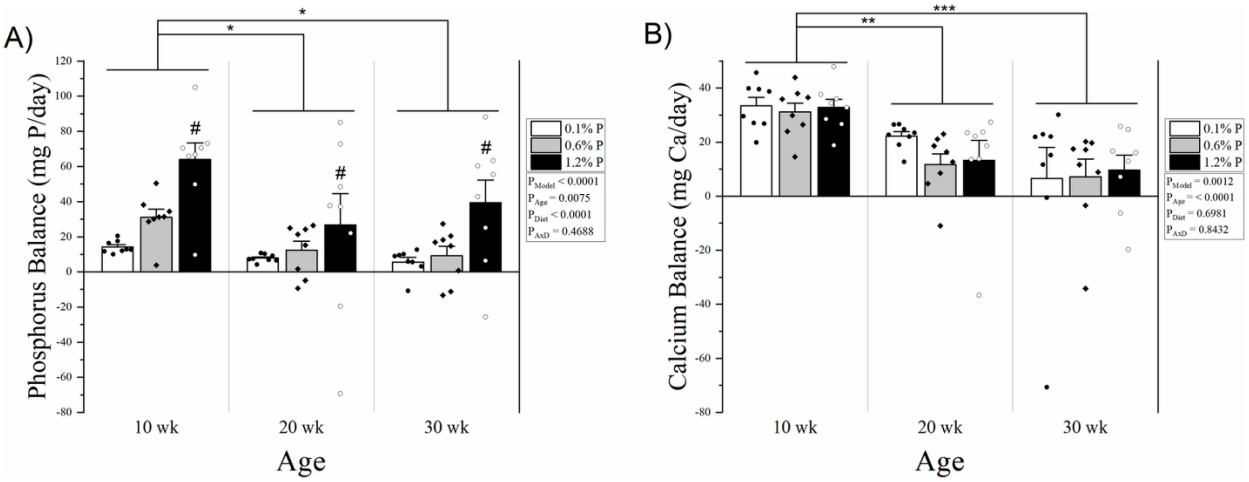


Figure 3.4. Phosphorus and calcium balance.
A) Phosphorus balance by age and dietary phosphorus intake level. There was a main effect for age where 10 week old rats had higher phosphorus balance compared to both 20 and 30 week olds, and the high phosphorus diet group was higher than normal and low phosphorus, with no significant age x diet interaction. B) Calcium balance by age and dietary phosphorus intake level. There was a main effect for age where 10 week old rats had higher calcium balance compared to both 20 and 30 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. Balance for each mineral was calculated as intake–fecal + urine. Means and standard error bars are shown for each group. Low phosphorus diet (0.1%) is shown in white bars and black dots; normal phosphorus diet (0.6%) is shown in grey with black diamonds; and high phosphorus diet (1.2%) is shown in black with white circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

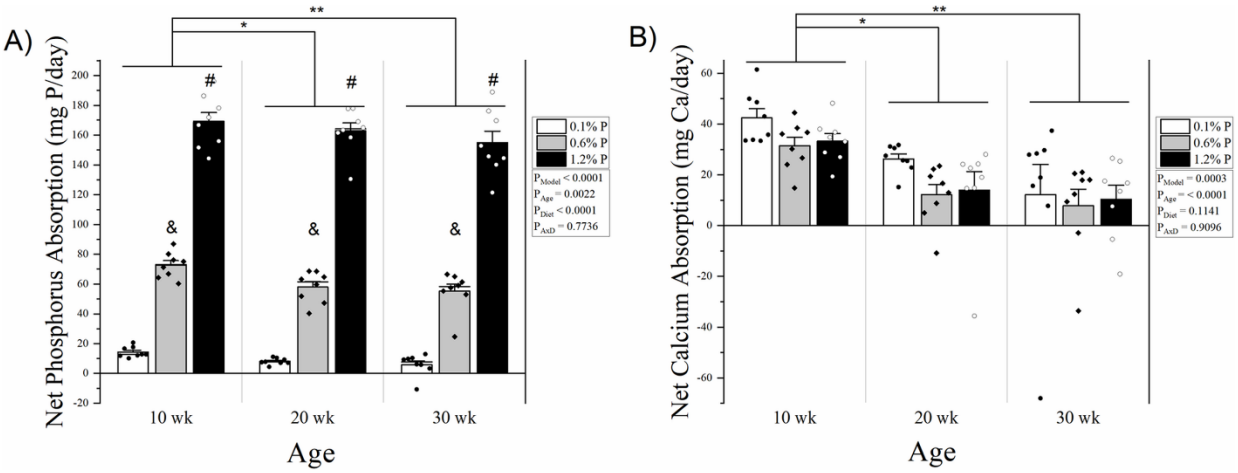


Figure 3.5. Net phosphorus and calcium absorption.

A) Net phosphorus absorption by age and dietary phosphorus intake level. There was a main effect for age where 10 week old rats had higher net phosphorus absorption compared to both 20 and 30 week olds, and net absorption increased with each phosphorus level in the diet, with no significant age x diet interaction. B) Net calcium absorption by age and dietary phosphorus intake level. There was a main effect for age where 10 week old rats had higher calcium balance compared to both 20 and 30 week olds, but there was no significant effect of dietary phosphorus intake level and no significant age x diet interaction. Net absorption for each mineral was calculated as intake–fecal. Means and standard error bars are shown for each group. Low phosphorus diet (0.1%) is shown in white bars and black dots; normal phosphorus diet (0.6%) is shown in grey with black diamonds; and high phosphorus diet (1.2%) is shown in black with white circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown. * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$ vs normal and low phosphorus, & $p < 0.0001$ vs high and low phosphorus.

Supporting Information

Supplemental Information for 33P Quench Curves

Plasma and intestinal ligated loop samples and standards were counted for ^{33}P radioactivity by liquid scintillation. Various constituents of these samples can cause both chemical and color quenching that artificially lowers counts per minute (CPM) detected by the liquid scintillation counter. Therefore, two quench curves were developed to correct for sample quench using the same scintillation cocktails as used for the samples. A standard quenching agent, nitromethane, was used to create the quench curve for use with the plasma samples, but in the case of the digested ligated loops, the sources of potential quench in the samples were simulated to create the quench curve. These quench curves were prepared based on methods described by Thomas (2014)(1).

Quench curve for plasma counting

Preparation of the quench curve

Thirteen replicate scintillation vials containing 15mL EcoLite scintillation cocktail (MP Biomedicals, Santa Ana, CA) and ~250,000 CPM ^{33}P (200 µL 0.5 µCi/mL ^{33}P-orthophosphate (Perkin Elmer, Waltham, MA) in deionized water) were prepared. Vials were counted on a Tri-Carb 2910 TR Liquid Scintillation Counter (Perkin Elmer, Waltham, MA) in a counting energy window of 0-300 keV for 10 minutes per vial (energy of ^{33}P: 76.4 keV average, 248.5 keV maximum) and CPM values obtained. The two vials that deviated the most from the mean CPM were discarded. The remaining 11 vials had mean radioactivity counts of 258,447 CPM and CV = 0.3%. These 11 vials were used to prepare a quench curve using nitromethane (Fisher Scientific, Hampton, NH) as a quenching agent in increasing amounts (**Table S3.1**). The quench parameter tSIE/AEC is determined from an external ^{133}Ba gamma source within the instrument, which is described in further detail in Thomas (2014)(1).

Results

The quench values (tSIE/AEC) and counting efficiencies (%) are presented in **Table S3.1**, and the quench curve is plotted in **Figure S3.1**. Unquenched ^{33}P (200 µL 0.5 µCi/mL solution in 15

mL scintillation cocktail) had a 98% counting efficiency. Counting efficiency was above 95% until the quench parameter tSIE/AEC reached ~200 (vial #7 =197 tSIE/AEC). Counting efficiency remained relatively high (above 70%) even with very high quench values seen in vials #9 and #10, and was only reduced to 59% in the most quenched (tSIE/AEC = 37) vial #11. Plasma samples analyzed in the present rat study had quench values in the range of ~470-490 tSIE/AEC which placed them on the quench curve at > 98% counting efficiency. Thus, correcting for counting efficiency only resulted in minor changes in radioactivity values of the rat plasma samples. Statistical analyses of data before and after quench curve corrections produced similar results for ANOVA model effects and group differences, but quench correction slightly increased group means.

Quench curve for intestinal ligated loop counting

Preparation of the quench curve

The present rat study utilized an *in situ* jejunal ligated loop method for phosphorus absorption assessment. Percent phosphorus absorption efficiency was calculated as:
$1 - (^{33}P$ activity remaining in digested loop) / (Total ^{33}P activity in 0.5mL dose) · 100. To determine the ^{33}P activity remaining in digested loop, the ~5cm excised intestinal segment was digested in 6 mL Soluene 350 (PerkinElmer, Waltham, MA) in a 45ºC oven, then divided into two scintillation vials and the color was lightened with the addition of 0.6mL of 30% hydrogen peroxide into each of the two vials for the purpose of reducing color quench. Total ^{33}P activity remaining in the digested loop was calculated by adding the counts from the two vials with the split sample. Therefore, an appropriate quench curve for the digested intestinal loops would simulate the constituents in the vials that are potential sources of quench. These factors include: intestinal tissue, thread from ligatures used to tie off the loop segment, absorption buffer solution injected into the ligated loop during the absorption test.
A range of these factors below and above the actual amounts and volumes utilized in the study were used in creating a quench curve. Intestinal tissue, thread, and solutes were added to nine scintillation vials (see lengths and volumes in **Table S3.2**) and left to digest for 3 days in an oven at 45ºC. Approximately 85,000 CPM ^{33}P (50 μL 0.8 μCi/mL ^{33}P-orthophosphate (Perkin Elmer, Waltham, MA) in deionized water) was added into each vial. Hydrogen peroxide and Hionic

Fluor scintillation cocktail (PerkinElmer, Waltham, MA) were added to each vial in amounts shown in **Table S3.2**. Vials were counted on a Tri-Carb 2910 TR Liquid Scintillation Counter in an open energy window of 0-2000 keV for 30 minutes per vial. The quench parameter tSIE/AEC was obtained from the counter output.

Results

The quench values (tSIE/AEC) and counting efficiencies (%) are presented in **Table S3.2**, and the quench curve is plotted in **Figure S3.2**. Unquenched ^{33}P (50 μL 0.8 μCi/mL solution in 20 mL scintillation cocktail) had > 99% counting efficiency. Counting efficiency declined to 89% with the addition of tissue, thread, and solutes. Using the same amounts of these factors as the used for the actual study samples ("normal conditions", vials 4 and 5), counting efficiency was 87% with a quench value of 196 tSIE/AEC. As shown in **Figure S3.2**, two vials (open blue circles on figure, vials 6 and 8) were excluded because their counting efficiencies did not follow the expected trend based on the curve established by the adjacent vials.
Intestinal loop samples analyzed in the current study had quench values in the range of ~150-230 tSIE/AEC, which placed them on the quench curve ~85-88% efficiency. Thus, correcting for counting efficiency based on the quench curve was important for reporting accurate intestinal phosphorus absorption values. Statistical analyses of data before and after quench curve corrections produced similar results for ANOVA model effects and group differences, but quench correction increased group means.

Conclusions

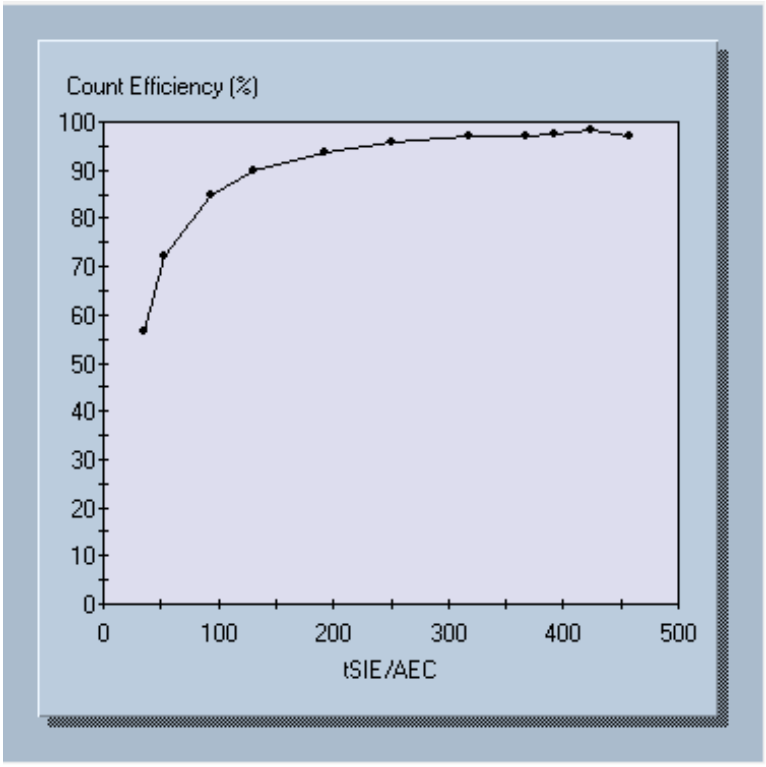
Counting efficiency by liquid scintillation counting is relatively high for ^{33}P for both plasma and digested intestinal ligated loop segments. However, the quench curves developed for the two types of samples were not interchangeable when comparing the tSIE/AEC values and associated % counting efficiencies on each curve (e.g. on the Ecolite/nitromethane curve, a quench value of 197 tSIE/AEC had a counting efficiency of 95%, but on the Hionic Fluor/Loop curve, a quench values of 195 tSIE/AEC had a counting efficiency of only 87%. Therefore, not only would unadjusted ^{33}P activity in digested ligated loops underestimate actual radioactivity in a sample, utilizing the wrong quench curve could also produce an erroneous result. This underscores the

importance of utilizing a quench curve that simulates as closely as possible the conditions of the samples to be counted (1).

Table S3.1. 33P quench curve with EcoLite scintillation cocktail for plasma counting

Vial #	Amount (µL) of nitromethane	tSIE/AEC	Count Efficiency (%)
1	0	468.63	98.22
2	5	435.48	99.83
3	10	401.87	98.84
4	15	378.68	98.63
5	26	325.32	98.66
6	45	258.69	96.55
7	70	197.87	95.58
8	110	134.92	90.84
9	150	97.21	86.69
10	230	55.73	73.77
11	310	37.89	59.63

Figure S3.1. Quench curve of 33P in EcoLite scintillation cocktail for plasma counting.



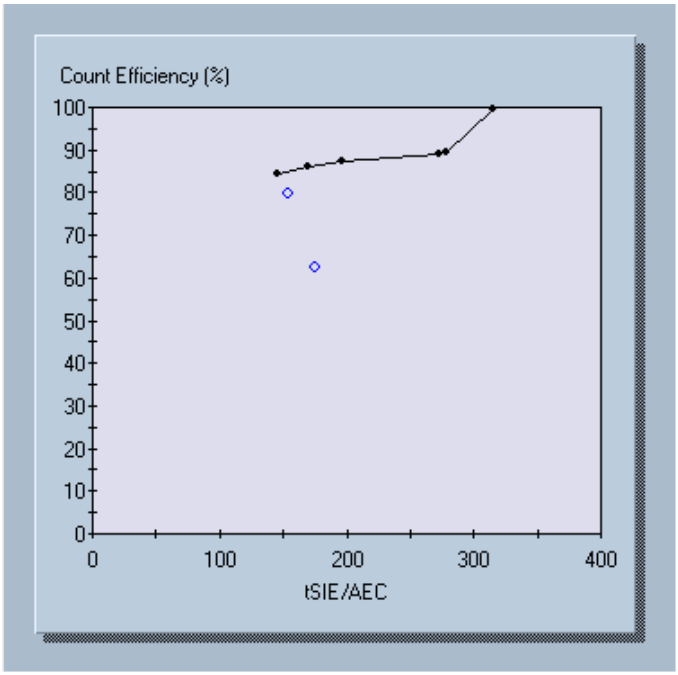
1. Thomson J. Internet: https://www.perkinelmer.com/liquidscintillation/images/APP_Use-and-Preparation-of-Quench-Curves-in-LSC_tcm151-171749.pdf.

Table S3.2. 33P quench curve with Hionic Fluor scintillation cocktail for loop counting

Vial #	Intestinal Segment Length (cm)	Ligature Thread (cm)	Soluene 350 vol. (mL)	Buffer vol. (10x dil.) (µL)	Hionic Fluor vol. (mL)	Hydrogen peroxide (mL)	tSIE/AEC	Count Efficiency (%)
1	0	0	0	0	20	0	315.13	99.78
2	1	0.5	1	50	19	0.1	278.96	89.30
3	1.5	1	1.5	50	18.5	0.2	272.47	89.05
4 ("normal conditions")	2.5	1.5	3	200	17	0.6	195.98	87.48
5 ("normal conditions")	2.5	1.5	3	200	17	0.6	195.85	87.25
6	3	2	3.5	250	16.5	0.7	174.52	62.86
7	3.5	2	3.5	250	16.5	0.8	169.07	85.96
8	4	3	4	300	16	0.8	153.06	80.22
9	4.5	3.5	4	350	16	0.8	145.43	84.24

Vials deemed outliers are indicated here in red text.

Figure S3.2. Quench curve of 33P in Hionic Fluor scintillation cocktail for loop counting.



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CHAPTER 4: EFFECT OF KIDNEY DISEASE PROGRESSION ON INTESTINAL PHOSPHORUS ABSORPTION AND PHOSPHORUS BALANCE IN MALE RATS

Manuscript draft intended for submission to the *Journal of Bone and Mineral Research*

Abstract

The Cy/+ rat has been characterized as a progressive model of chronic kidney disease-mineral bone disorder (CKD-MBD). We aimed to determine the effect of kidney disease progression on intestinal phosphorus (P) absorption and whole-body P balance in this model. N=48 Cy/+ (CKD) and N=48 normal littermates (WT) rats were studied at two ages: 20wk and 30wk, to model progressive kidney decline. Sodium-dependent and sodium-independent intestinal P absorption efficiency was measured by *in situ* jejunal ligated loops using ^{33}P radioisotope. Our results show that CKD rats had slightly higher sodium-dependent absorption compared to WT rats, and absorption decreased from 20 to 30 weeks. These results are in contrast to measured $1,25OH_2D$, which was lower in CKD rats. Gene expression of the major intestinal phosphate transporter, NaPi-2b, was not different between groups in the jejunum, but lower in CKD rats in the duodenum. Ligated loop absorption results are supported by an higher percent net phosphorus absorption in CKD rats from metabolic balance and higher in 20wk olds versus 30wk olds. Phosphorus balance was more negative in CKD rats compared to WT and higher in 20wk olds versus 30wk olds. These results demonstrate no reduction in intestinal phosphorus absorption with progression of CKD despite a decrease in $1,25OH_2D$ status when assessed by an *in situ* ligated loop test.

Introduction

Chronic kidney disease-mineral bone disorder (CKD-MBD) is characterized by biochemical abnormalities related to calcium and phosphorus metabolism, including elevated fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), and serum phosphorus, and lower serum 1,25-dihydroxyvitamin D3 (1,25D) and calcium, bone abnormalities, and vascular or other soft tissue calcification (1). Phosphorus and calcium regulating hormones (FGF23, 1,25D, PTH) change in early stages of the disease to maintain serum mineral concentrations (2, 3). However, these hormonal alterations have secondary consequences that contribute to the elevated risk for cardiovascular events, bone fragility fractures, and death (4-6). Because phosphate dysregulation is a central driver of these adverse events, interventions aimed at maintaining phosphate homeostasis have been of interest, including targeting the intestinal absorption of dietary phosphorus (7).

Gaps exist in understanding the hormonal regulation of intestinal phosphate absorption in CKD. 1,25D is a recognized positive regulator of the main known intestinal phosphate transporter, sodium phosphate cotransporter-2b (NaPi-2b) and active intestinal phosphate absorption (8). In CKD, 1,25D is suppressed via elevated FGF23 (9), and therefore active intestinal phosphate absorption is expected to be lower as kidney function declines. However, literature on this topic is mixed. In humans, reduced phosphorus absorption has been demonstrated in patients with end-stage renal disease using metabolic balance studies as well as a radioisotopic phosphorus tracer (10-12) and in patients on hemodialysis by a triple-lumen perfusion technique (13). These findings have been supported in some but not all studies in experimental rat models: Peerce et al. (14) showed decreased sodium-dependent jejunal brush border membrane vesicle (BBMV) uptake in 5/6 nephrectomized rats versus age-matched controls, and Moe et al. (15) found a reduction in active phosphate transport by Ussing chamber in Cy/+ CKD rats compared to normal rats, though this effect appeared to be driven by rats treated with phosphate binders. In contrast, sodium-dependent jejunal BBMV uptake was not different in 5/6 nephrectomized rats versus sham-operated rats in two additional studies (16, 17). Marks et al. (18) also found no difference in 5/6 nephrectomized versus sham-operated rats using the *in situ* ligated loop method in the jejunum or duodenum, and gene expression of the major intestinal phosphate transporter, NaPi-2b, was also not downregulated with 5/6 nephrectomy.

Further, in rats with adenine-induced CKD, the mild-CKD and CKD rats had similar appearance of ^{33}P into serum over 2 hours after an oral gavage compared with controls (19). It is unclear whether differences in these rat experiments are the result of different stages of severity of the disease, or methodological differences in absorption assessment technique. This question is important to resolve, because if intestinal phosphorus absorption is in fact reduced with disease, approaches targeting active intestinal phosphorus transport may be less effective than anticipated. If intestinal phosphorus absorption is not reduced with disease, then this suggests further complexity than described in current models of phosphorus regulation in CKD.

There are additional unanswered questions in regard to phosphate homeostasis with CKD progression. Given that changes to tight junction proteins are observed in CKD (20, 21), and given that paracellular transport of intestinal phosphate is not well understood, it is plausible that sodium-independent absorption could also change with disease progression. To our knowledge, only one study has assessed this using BBMV uptake and found no difference in sodium-independent absorption with CKD compared to controls (17). Additionally, it is unclear whether whole-body phosphorus retention increases with CKD progression, as a driver or consequence of disease. Certainly, elevated serum phosphate appears in later stages of disease, but serum phosphate does not necessarily reflect whole-body status. Hill et al. showed an average whole-body phosphorus balance in moderate-stage CKD patients was around zero and was not affected by a calcium-based phosphate binder (22), but large variability existed with some patients having negative, neutral, or position phosphorus balance (23).

In this study we aimed to determine the effects of kidney disease progression in the Cy/+ rat model of progressive kidney decline (24) on intestinal phosphorus absorption as measured by the *in situ* ligated loop absorption method, as well as whole-body phosphorus balance, biochemistries of phosphorus and calcium metabolism, and gene expression of the major intestinal phosphate transporters in the jejunum and duodenum. We hypothesized that absorption would be maintained at a moderate stage of kidney disease but decreased at the later stage, corresponding to a reduction in NaPi-2b expression, and also lower phosphorus balance.

Materials and Methods

Animals

Ninety-six total male rats were obtained from a Cy rat colony at the Indiana University School of Medicine. Forty-eight heterozygous Cy/+ (CKD) and forty-eight wild-type +/+ (WT) litter-match controls were randomly assigned to 20- or 30-week-old age groups in a 2x2 factorial design (N = 24 rats per age x genotype). Half of the rats in each group were randomly assigned to the sodium-dependent absorption test outcome or the sodium-independent absorption test outcome (n = 12 rats/age x buffer group). Rats were fed standard rat chow containing 0.7% phosphorus and 1.0% Ca (Envigo Teklad 2018, Madison, WI) and water *ad libitum* until 16 weeks of age, at which time they were switched to an *ad libitum* casein-based diet (0.7% phosphorus and 0.7% calcium) that has been shown to accelerate kidney decline (TD.04539, Envigo Teklad, Madison, WI) (24) until sacrifice. Rats were housed individually in shoe-box cages until five days prior to sacrifice when they were transferred to wire-bottom metabolic cages and phosphorus and calcium balance was performed during the last four days prior to sacrifice. Body weights were taken weekly. The light-dark cycle was maintained from 6AM-6PM. This protocol was approved by the Purdue University Animal Care and Use Committee.

Intestinal Phosphorus Absorption Efficiency

Intestinal phosphorus absorption efficiency was determined by *in situ* jejunal ligated loop absorption tests as described previously (25) with the exception of being fasted on the day of sacrifice from midnight until morning for the ligated loop absorption test. Half (N = 12) of the rats in each age x genotype group were randomly assigned to the "sodium-dependent" absorption test using a transport buffer containing (mmol/L): 16 Na-N-2-hydroxyethylpiperazine-N0-2-ethanesulfonicacid or 4-(2-Hydroxyethyl)piperazin-1-ylethanesulfonic acid, 140 NaCl or ChCl, 3.5 KCl, 0.1 KH2PO4, and ~5 uCi ^{33}P (^{33}P-orthophosphoric acid, PerkinElmer, Waltham, MA), pH 7.4, and the other half (N = 12) to the "sodium-independent" absorption test using a transport buffer containing (mmol/L): 4-(2-Hydroxyethyl)piperazin-1-ylethanesulfonic acid, 140 ChCl, 3.5 KCl, 0.1 KH2PO4, and ~5 uCi ^{33}P, pH 7.4. After injection of the transporter buffer, blood (0.5 mL/sampling) was collected at 5, 10, 15, and 30 min post-injection in lithium heparin tubes and

centrifuged at 5000 RPM for 10 minutes (Labofuge A 2502, Baxter Scientific Products, McGaw Park, IL) to separate plasma. Jejunal loops were removed and heated until dissolved (up to 3 days) at 45^{o}C in an oven.

Absorption of ^{33}P was evaluated two ways: 1) area under the curve (AUC) was calculated for plasma ^{33}P activity over 30 minutes, and 2) percent intestinal phosphorus absorption efficiency over 30 minutes was calculated as:

$1 - (^{33}\text{P}$ activity remaining in digested loop) / (Total ^{33}P activity in 0.5mL dose) $\cdot$ 100

In addition, because absorption without sodium in the buffer may not be truly sodium-independent (26), an "exclusively" sodium-dependent component was calculated from each rat as:

(^{33}P absorption (from loop or AUC)) – (average absorption for the corresponding rat's group given the absorption buffer without sodium)

Phosphorus and Calcium Balance and Net Absorption

Over the four days prior to sacrifice, urine and feces were collected per (25) to assess balance and net absorption of phosphorus and calcium. Feces and diet were ashed in a muffle furnace (Thermolyne Sybron Type 30400, Dubuque, IA) for 10 days at 600^{o}C, and diluted 1400X and diet 140X with 2% nitric acid. Urine was diluted 11X with 2% nitric acid. Phosphorus and calcium in urine, feces, and diet were quantified by inductively coupled plasma-optical emission spectrophotometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT). Urine creatinine was determined by colorimetric method (Quantichrom, BioAssay Systems, Hayward, CA). Four-day phosphorus balance was calculated as dietary phosphorus intake (mg/d) – urine and fecal phosphorus excretion (mg/d), and net phosphorus absorption (%) as phosphorus intake (mg/d) – fecal excretion (mg/d) / phosphorus intake (mg/d) $\cdot$ 100. Calcium balance and net calcium absorption were calculated similarly.

Intestinal Phosphate Transporter Gene Expression

After the completion of the ligated loop absorption tests and the removal of the radioactive jejunal loop, approximately 8 cm of jejunum distal to the ligated loop and the duodenum distal to the pylorus up to the ligated loop were removed and cut open. The mucosal layers were scraped, and mucosa from each intestinal segment was placed into TRI Reagent

(Fisher Scientific, Hampton, NH) and flash frozen in liquid nitrogen for later mRNA quantification by RT-PCR. The left kidney was removed, and flash frozen in foil for later mRNA quantification. Upon thawing, kidneys were weighed and cut into thirds cross-sectionally. The cortex was removed, mixed, and a sample placed into TRI Reagent (Fisher Scientific, Hampton, NH).

Gene expression of intestinal NaPi2b and PiT1 using real-time PCR was performed as previously described (25). NaPi2a and NaPi2c were assessed in the renal cortex (Applied Biosystems Rn00564677_m1 and Rn00595128_m1).

Plasma Biochemistries

Plasma stored at -80C was thawed and analyzed for phosphorus, calcium, creatinine, and blood urea nitrogen (BUN) concentration by colorimetric methods (phosphorus and calcium: Pointe Scientific, Inc., Canton, MI; creatinine and BUN: Quantichrom, BioAssay Systems, Hayward, CA). Intact PTH (iPTH) and intact FGF23 (iFGF23) were measured by enzyme-linked immunosorbent assay (Quidel, San Diego, CA), and 1,25D by enzyme immunoassay (Immunodiagnostic Systems, The Boldons, UK).

Statistics

A sample size of n = 12 rats/group was determined to be sufficient to detect a 30% difference between groups for phosphorus absorption ($\beta = 0.80$, $\alpha = 0.05$) based on means and standard deviations reported by Marks et al. (18). Two-way ANOVA was performed for all outcomes with main effects for age and genotype and their interaction, with Tukey post-hoc comparisons. Statistical significance was set at $\alpha < 0.05$. Statistical Analysis Software version 9.4 (SAS Institute, Cary, NC) was used for all statistical analysis. Results are reported as mean ± SEM unless otherwise indicated.

Results

At 16 weeks of age (baseline), plasma creatinine was higher in CKD rats compared with WT (**Table 4.1**). Final creatinine was higher in CKD rats, while change from baseline was higher in CKD rats compared with WT and in 30 week old rats compared with 20 week old rats (**Table**

4.2). Urine creatinine was higher in 30 week olds compared with 20 week olds (**Table 4.2**). Creatinine clearance and hematocrit were lower in CKD rats compared with WT but not different between age groups (**Table 4.2**). There was an interaction for kidney weight where 30 week old CKD rats were higher than all other groups ($p < 0.0001$ for all), and 20 week old CKD rats were higher than WT 20 week olds and 30 week olds ($p < 0.0001$ and $p = 0.0026$, respectively) (**Table 4.2**).

iFGF23 at basline was higher in the CKD rats compared with WT (**Table 4.1**). An age x genotype interaction existed for final and change from baseline in iFGF23, where 30 week old CKD rats higher final concentrations and greater increase from baseline than all other groups ($p < 0.0001$ for all post-hoc comparisons, **Table 4.2**). Baseline iPTH was not different between CKD and WT (**Table 4.1**). Final levels and change from baseline in iPTH were higher in 30 week old CKD rats versus other groups (**Table 4.2**). Baseline 1,25D was lower in CKD rats compared with WT (**Table 4.1**). There was a significant effect for main effects of age and genotype for final 1,25D, where CKD was lower than WT, and 30-week-olds were lower than 20-week-olds. However, Tukey's post-hoc tests revealed the only difference in pairwise comparisons was for 30-week-old CKD rats that had lower 1,25D than all other groups, despite a non-significant $p = 0.06$ for the interaction (**Table 4.2**).

With the "sodium-dependent" absorption test, phosphorus absorption efficiency from loops at 30 minutes was higher in 20 week old rats compared with 30 week olds (35.7 ± 0.9 % vs 27.7 ± 1.3 %, $p < 0.0001$), and higher in CKD rats compared with WT (33.4 ± 1.4 % vs 30.0 ± 1.3 %, $p = 0.0283$, interaction 0.6913) **(Figure 4.1)**. With the "sodium independent" absorption test, there was a significant age x genotype interaction ($p = 0.0149$), where 20 week old CKD and WT rats had higher absorption efficiency than 30 week old WT rats (24.5 ± 1.6 % and 27.3 ± 1.5 % vs 18.5 ± 1.3 %, $p = 0.0197$ and $p = 0.0003$) **(Figure 4.1)**. Phosphorus absorption by plasma AUC had a similar pattern; in the "sodium dependent" test, absorption was higher in 20 week old rats compared with 30 week olds (0.7 ± 0.04 vs 0.5 ± 0.03, $p < 0.0001$), and higher in CKD rats compared with WT (0.7 ± 0.03 vs 0.5 ± 0.04, $p = 0.0008$; interaction $p = 0.5769$) **(Figure 4.2)**. In the "sodium independent" test, absorption was higher in 20 week old rats compared with 30 week old rats (0.5 ± 0.03 vs 0.4 ± 0.02, $p = 0.0011$), and higher in CKD compared with WT (0.5 ± 0.02 vs 0.4 ± 0.02, $p < 0.0001$; interaction $p = 0.9664$) **(Figure 4.2)**. There was an age x genotype interaction for "exclusively" sodium-dependent absorption

efficiency from the ligated loops (p = 0.0189) where 20 week old CKD rats had higher absorption efficiency compared with 20 week old WT and 30 week old CKD rats (13.2 ± 1.0 % vs 7.1 ± 1.5 % and 6.3 ± 1.9 %, p = 0.0376 and p = 0.0152) **(Figure 4.3A)**. For absorption by plasma AUC, sodium-dependent absorption was not different between groups (overall model p = 0.3486) **(Figure 4.3B)**. Body weight at sacrifice was lower in CKD rats compared with WT and in 20 week olds vs 30 week olds **(Table 4.2)**, however assessment of absorption with body weight as a covariate in models of loops or plasma did not change results (data not shown). The sodium-dependency of phosphate absorption varied from 22-35% for CKD rats and 19-33% depending on age and method of determination (ie label disappearance from loop or appearance into plasma) (**Table 4.3**).

NaPi-2b mRNA did not differ by groups in the jejunum, but in the duodenum it was lower in CKD rats compared with WT, while there was no difference between 20 and 30 week olds **(Figure 4.6)**. There were no effects of age or genotype on PiT-1 mRNA in either intestinal segment **(Figure 4.6)**. In the kidney, both NaPi-2a and NaPi-2c were lower in CKD rats compared with WT but there was no difference between age groups **(Figure 4.6)**.

Phosphorus balance was higher in 20 week olds compared with 30 week olds (0.94 ± 2.2 mg/d vs -12.7 ± 3.2 mg/d, p = 0.0005), and higher in WT compared with CKD rats (-2.2 ± 2.8 mg/d vs -9.7 ± 3.0 mg/d, p = 0.0470), with no age x genotype interaction (p = 0.7881) (**Figure 4.4A**). Calcium balance was higher in 20 week olds compared with 30 week olds (17.8 ± 2.6 mg/d vs 7.3 ± 1.7 mg/d, p < 0.0001), while there was no difference between WT and CKD groups (11.3 ± 1.9 mg/d vs 13.9 ± 1.7 mg/d, p = 0.2924), and no age x genotype interaction (p = 0.5889) (**Figure 4.4B**). Net phosphorus balance was higher in 20 week olds compared with 30 week olds (90.8 ± 1.5 mg/d vs 76.9 ± 1.9 mg/d, p < 0.0001), but was not different between WT and CKD rats (81.6 ± 1.8 mg/d vs 86.2 ± 2.1 mg/d, p = 0.0759), interaction p = 0.7898 (**Figure 4.5A**). Net calcium balance was also higher at 20 weeks compared with 30 weeks (20.7 ± 1.6 mg/d vs 11.9 ± 1.7 mg/d, p = 0.0001), but not different between WT and CKD (14.0 ± 1.8 mg/d vs 18.2 ± 1.7, p = 0.0848), interaction p = 0.4988 (**Figure 4.5B**). Individual components of balance are listed in **Tables 4.4 and 4.5**.

Discussion

In this study, 20-week-old rats had higher intestinal phosphorus absorption efficiency compared with 30-week-old rats as measured by *in situ* jejunal ligated loop. This corresponds with the higher net phosphorus absorption from metabolic balance and greater positive overall whole-body phosphorus balance observed in 20-week-old versus 30-week-old rats. Interestingly, these results are in contrast with our previous study in healthy Sprague Dawley male rats, in which we observed no age difference in jejunal phosphorus absorption efficiency, net phosphorus absorption, nor balance between 20-week-old and 30-week-old rats using similar methods (25). This may be due, however, to a difference in rat strain. The Cy rats obtained from the inbred IUSM colony have a Han:Sprague Dawley background (27), which may have different physiologic adaptations with age compared to commercial Sprague-Dawley rats (Envigo). However, in both studies, the age effects on absorption mirrored the age effects on 1,25D: no difference in 1,25D or absorption in 20-week-old versus 30-week-old Sprague-Dawley rats; higher 1,25D and higher absorption in 20-week-old versus 30-week-olds in the present study. This is in *agreement* with the classic understanding of a positive relationship between 1,25D and intestinal phosphorus absorption.

Notably, in CKD rats, intestinal phosphorus absorption efficiency was slightly but statistically higher compared with WT, which runs in *contrast* to the lower 1,25D levels in CKD compared with WT. Similarly, net phosphorus absorption from metabolic balance was not statistically different between CKD and WT ($p = 0.08$) but was numerically higher in CKD, in line with the ligated loop findings. A lack of decrease in absorption is supported by the work of others using in situ or in vivo absorption assessment methods. Marks et al. (28) observed no statistical difference in phosphorus absorption using the ligated loop in 5/6 nephrectomized CKD rats in either the jejunum or duodenum (but numerically higher in the CKD rats), despite lower 1,25D in CKD rats. Recently, Turner et al. (19) also showed no difference in phosphorus absorption in adenine-induced moderate or mild CKD rats compared to controls using an *in vivo* oral gavage method. These data and ours suggest that CKD does not result in a physiological adaptation to limit intestinal phosphorus absorption even when 1,25D is low.

In vitro studies contrast in jejunal brush border membrane vesicle (BBMV) uptake in 5/6 nephrectomized rats finding either decreased uptake compared with age matched controls or no change compared with sham surgery (16, 17). Interestingly, despite showing a reduction in

sodium-dependent uptake, Peerce et al. found no difference in the percentage of phosphorus absorbed from metabolic balance in CKD rats (14). Further, Marks et al. observed no difference in uptake as assessed by the everted gut sac technique in 5/6 nephrectomized rats compared with sham-operated (28). In vitro techniques assess uptake into enterocytes, whereas the in situ and in vivo assessments include basolateral transport into circulation. These methodological differences could illuminate whether basolateral regulation can explain different outcomes, although the inconsistencies within in vitro results generally rules this out.

We observed some differences when assessing phosphate transport in an absorption buffer without sodium, although it unclear whether these reflect true sodium-independency with the ligated loop technique. Luminal phosphate concentration in vivo is ~1.5-40 mM in the proximal intestine depending on measurement technique (26, 29), which would result in higher passive transport compared to in vitro techniques, despite utilizing a low phosphate concentration in the loop. Further, endogenous sodium secretion during the test may contribute to sodium-dependent absorption (26). Thus, it is unlikely that we are able to observe true sodium-independent absorption with the ligated loop. This is also reflected in the much lower estimations of sodium-dependency when using the ligated loop or oral gavage (26, 30). Our finding of sodium-dependency estimated by appearance into plasma in WT rats of 33% is in alignment of the finding of 32% by Marks et al. in healthy Sprague Dawley rats (26). Loghman-Adham et al found no change in sodium-independent uptake in CKD (17). Changes in tight gap junction proteins occur in 5/6 nephrectomy- or adenine- induced CKD along the intestine, although the relevance of these to intestinal phosphate transport is unclear (20, 21).

We observed no difference in NaPi-2b mRNA between CKD and WT rats in the jejunum, but lower NaPi-2b in CKD rats compared with WT in the duodenum. No age or CKD differences in PiT-1 mRNA were observed in either intestinal segment. Similarly, Marks et al. observed no difference in either duodenum nor jejunum NaPi-2b mRNA expression between 5/6th nephrectomized compared with sham-operated rats (18). However, we have previously (15) observed lower NaPi-2b mRNA in the duodenum and jejunum, but not ileum, in the same Cy/+ rat model compared with WT controls of similar age to the present study. We observed more extreme elevations in FGF23 and lower 1,25D in the CKD rats in the prior study, which may explain the discrepancy in findings between studies in regard to NaPi-2b mRNA expression.

However, it is notable in both studies, the absorption outcomes generally reflected NaPi-2b mRNA expression outcomes.

While patients with end-stage renal disease (10-12) and on dialysis (13) appear to have reduced absorption of dietary phosphorus compared to healthy individuals, whether it is reduced in moderate stages of the disease is unresolved. Our results, together with others suggest that absorption is relatively unchanged as the disease progresses to later stages in animal models. Thus, interventions that target active intestinal phosphorus transport may be fruitful. Although 1,25D decreases markedly with disease progression, we observed no reduction in phosphate absorption, despite its well-documented positive regulation of absorption via NaPi-2b. Future work could assess whether a threshold exists or if additional regulation is maintaining phosphate transport in CKD.

Table 4.1. Baseline blood biochemistries

	20 weeks old		**30 weeks old**		**P-Values**
	WT	CKD	WT	CKD	Genotype
Plasma Creatinine (mg/dL)	0.39 (0.02) n=18	0.55 (0.01) n=18	0.30 (0.02) n=14	0.51 (0.03) n=19	**<0.0001**
Plasma PTH (pg/mL)	376.7 (38.9) n = 15	349.3 (31.8) n = 15	341.3 (29.5) n = 15	339.6 (23.2) n = 16	0.6325
Plasma iFGF23 (pg/mL)	441.6 (21.1) n = 15	745.1 (26.6) n = 15	375.9 (8.8) n = 15	699.5 (31.2) n = 16	**<0.0001**
Plasma 1,25D (pg/mL)	231.7 (61.9) n = 14	85.9 (22.9) n = 14	205.8 (55.0) n = 14	101.6 (26.2) n = 15	**<0.0001**

†**Baseline blood and urine biochemistries**. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown, and means and (SEM) are shown for each group. Plasma creatinine was higher at 20 weeks vs 30 weeks and higher in CKD vs WT. Plasma PTH did not differ between groups. iFGF23 was higher at 20 weeks vs 30 weeks and higher in CKD rats vs WT. Plasma 1,25D was lower in CKD vs WT.

Table 4.2. Final weights and blood and urine biochemistries†

	20 weeks old		**30 weeks old**		**P-Values**			
	WT	CKD	WT	CKD	Model	Age	Genotype	Age x Genotype
Body weight (g)	487.9 (5.4) n = 24	459.5 (5.9) n = 25	546.3 (7.7) n = 24	511.0 (5.9) n = 23	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.5814
Kidney weight (g)	1.7 (0.4) n = 22	2.1 (0.4) n = 22	1.8 (0.4) n = 24	2.7 (0.6) n = 23	**< 0.0001**	**<0.0001**	**<0.0001**	**0.0002**
Final Plasma Creatinine (mg/dL)	0.45 (0.02) n = 23	0.78 (0.03) n = 23	0.46 (0.02) n = 23	0.91 (0.06) n = 22	**<0.0001**	0.0740	**<0.0001**	0.1196
Change Plasma Creatinine (mg/dL)	0.06 (0.03) n = 17	0.25 (0.04) n = 18	0.15 (0.03) n = 13	0.33 (0.06) n = 18	**<0.0001**	**0.0451**	**<0.0001**	0.8960
Urine Creatinine (mg/day)	767.7 (88.3) n = 24	595.7 (31.4) n = 24	958.5 (72.2) n = 24	875.4 (56.5) n = 22	**0.0013**	**0.0006**	0.0565	0.5024
Hematocrit (%)	49.5 (10.1) n = 24	43.3 (9.2) n = 22	49.5 (10.1) n = 24	42.4 (8.8) n = 23	**<0.0001**	0.6008	**<0.0001**	0.6026

Table 4.2 cont

Creatinine Clearance (mL/min)	**2.55 (0.36) n = 24**	**1.18 (0.07) n = 24**	**2.74 (0.23) n = 23**	**1.46 (0.16) n = 22**	**<0.0001**	**0.3176**	**<0.0001**	**0.8318**
Plasma PTH (pg/mL)	839.7 (98.1) n = 24	1116.5 (122.7) n = 23	1246.8 (128.2) n = 24	1707.2 (176.7) n = 18	0.0002	0.0002	0.0058	0.4822
Change plasma PTH (pg/mL)	418.5 (118.6) n = 15	691.6 (115.8) n = 14	880.6 (150.0) n = 15	1301.52 (210.9) n = 14	0.0015	0.0009	0.0270	0.6303
Plasma iFGF23 (pg/mL)	527.0 (24.5) n = 24	841.0 (49.7) n = 24	538.9 (42.2) n = 24	2215.5 (287.5) n = 23	<0.0001	<0.0001	<0.0001	<0.0001
Change plasma iFGF23 (pg/mL)	81.7 (40.5) n = 15	138.5 (55.6) n = 15	126.9 (24.4) n = 15	1318.4 (322.9) n = 16	<0.0001	0.0008	0.0007	0.0018

Table 4.2 cont

Plasma 1,25D (pg/mL)	**278.6 (56.9) n = 24**	**259.2 (52.9) n = 24**	**232.9 (47.5) n = 24**	**93.6 (19.5) n = 23**	**0.0003**	**0.0013**	**0.0142**	***0.0621***
Change plasma 1,25D (pg/mL)	97.5 (26.0) n = 14	164.3 (43.9) n = 14	64.7 (17.3) n = 14	-0.2 (-0.06) n = 15	0.0765	0.0306	0.9830	0.1437

†Final weights, and blood and urine biochemistries. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown, and means and (SEM) are shown for each group. Body weights were lower at 20 weeks vs 30 weeks and lower in CKD rats vs WT. Kidney weight was higher in 30 week CKD rats. Plasma creatinine and change from baseline was higher in CKD rats vs WT, and it increased more in the 30 week rats. Urine creatinine was highest in 30 week rats vs 20 week. Hematocrit and creatinine clearance was lower in CKD rats vs WT. Plasma PTH and change from baseline was greater in CKD vs WT and at 30 weeks vs 20 weeks. iFGF23 and change from baseline was highest in the 30 week CKD rats. Plasma 1,25D was lower in CKD rats vs WT and lower at 30 weeks vs 20 weeks.

Table 4.3. Percent sodium-dependency of the jejunum by genotype and age

	20 Weeks		**30 Weeks**	
% sodium-dependency	**CKD**	**WT**	**CKD**	**WT**
Disappearance from loop (%)	35	19	22	29
Appearance into plasma (%)	25	33	25	22

Sodium-dependency was determined by (average absorption with buffer with sodium-average absorption with buffer without sodium/average absorption with buffer with sodium)

Table 4.4. Components of balance for phosphorus

Component	**20 weeks old**		**30 weeks old**		**P-Values**			
	WT	CKD	WT	CKD	Model	Age	Genotype	Age x Genotype
Balance (mg/d)	4.3 (3.5)	-2.4 (2.7)	-8.4 (4.0)	-17.2 (5.0)	**0.0015**	**0.0005**	**0.0470**	0.7881
Net absorption (mg/d)	11.2 (2.3)	93.3 (2.0)	75.1 (2.1)	78.8 (3.2)	**<0.0001**	**<0.0001**	0.0759	0.7898
Net absorption (%)	48.4 (1.1)	51.1 (0.9)	43.7 (1.1)	47.7 (0.8)	**0.0005**	**0.0012**	**0.0065**	0.6068
Fecal phosphorus (mg/d)	94.1 (3.3)	89.5 (2.6)	96.0 (2.1)	86.3 (3.4)	**0.0406**	0.8226	**0.0069**	0.3272
Urine phosphorus (mg/d)	84.0 (2.5)	95.7 (2.8)	83.5 (3.3)	96.0 (4.8)	0.0088	0.9749	0.0007	0.9096
Dietary phosphorus intake (mg/d)	182.4 (2.2)	182.8 (3.1)	171.1 (1.8)	165.1 (3.5)	**<0.0001**	**<0.0001**	0.2989	0.2394

ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown, and means and (SEM) are shown for each group.

Table 4.5. Components of balance for calcium

Component	**20 weeks old**		**30 weeks old**		**P-Values**			
	WT	CKD	WT	CKD	Model	Age	Genotype	Age x Genotype
Balance (mg/d)	17.2 (2.6)	18.4 (1.9)	5.5 (11.0)	9.2 (2.6)	**0.0002**	**<0.0001**	0.2924	0.5889
Net absorption (mg/d)	19.5 (2.5)	21.9 (1.9)	8.8 (2.2)	14.3 (12.5)	**0.0004**	**0.0001**	0.0848	0.4988
Net absorption (%)	13.8 (1.8)	15.6 (1.3)	6.6 (1.6)	11.1 (2.0)	**0.0017**	**0.0009**	0.0663	0.4212
Fecal calcium (mg/d)	121.4 (2.8)	119.2 (2.9)	123.4 (2.4)	113.2 (3.4)	0.0825	0.4924	0.0349	0.1668
Urine calcium (mg/d)	2.3 (0.5)	3.5 (0.2)	3.2 (0.2)	5.1 (0.4)	**<0.0001**	**<0.0001**	**<0.0001**	0.1696
Dietary calcium intake (mg/d)	140.9 (1.7)	141.1 (2.4)	132.1 (1.4)	127.5 (2.7)	**<0.0001**	**<0.0001**	0.2989	0.2394

ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet (P_{Diet}), and interaction of age and diet (P_{AxD}) are shown, and means and (SEM) are shown for each group.

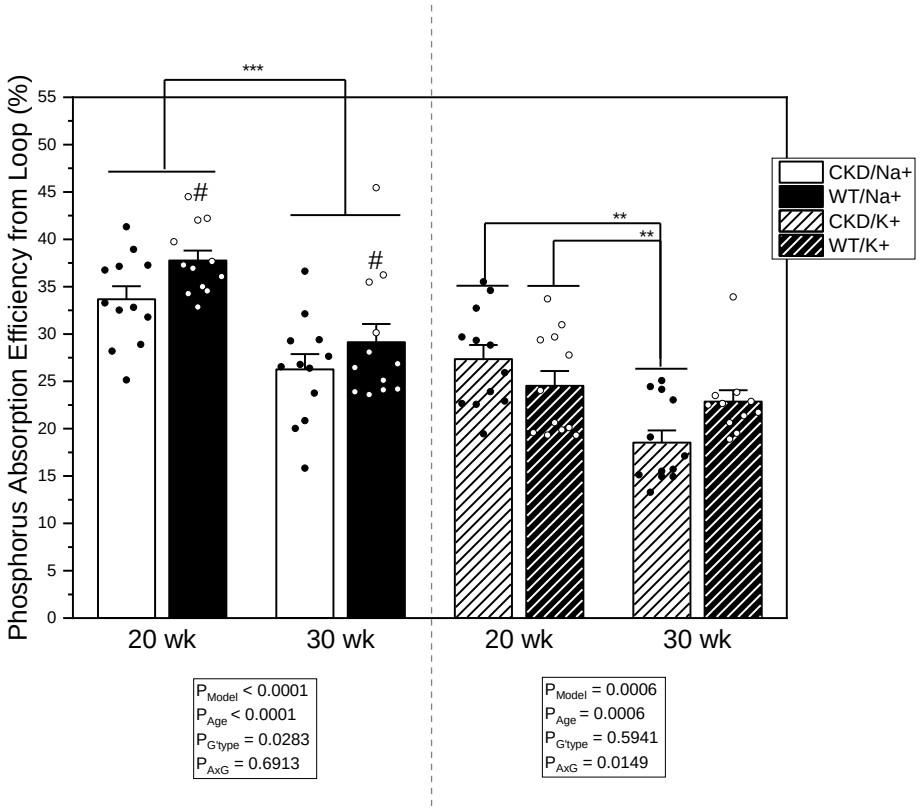

Figure 4.1. Percent jejunal phosphorus absorption efficiency by age (20 or 30 weeks) and genotype (CKD or WT) with or without sodium in the absorption buffer.
Phosphorus absorption efficiency was calculated as 1-(^{33}P activity remaining in jejunal loop)/(Total ^{33}P activity in dose) after 30 minutes post ^{33}P injection into the jejunal loop. Means and standard error bars are shown for each group. For rats given the absorption buffer with sodium (left), there was a main effect for age where 20 week old rats had higher absorption efficiency compared to both 30 week olds, and CKD rats had higher balance compared to WT, with no significant age x diet interaction. For rats given the absorption buffer without sodium (right), there was a significant age x genotype interaction, with 20 week old CKD and WT rats greater than 30 week WT rats. Absorption buffer with sodium: CKD rats are shown with black bars and white dots, WT rats are shown with white bars and black dots. Absorption buffer without sodium: CKD rats are shown with black bars with white hashes and white dots, WT rats are shown with white bars with black hashes and black dots. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown. ** $p < 0.01$, *** $p < 0.0001$, # $p < 0.05$.

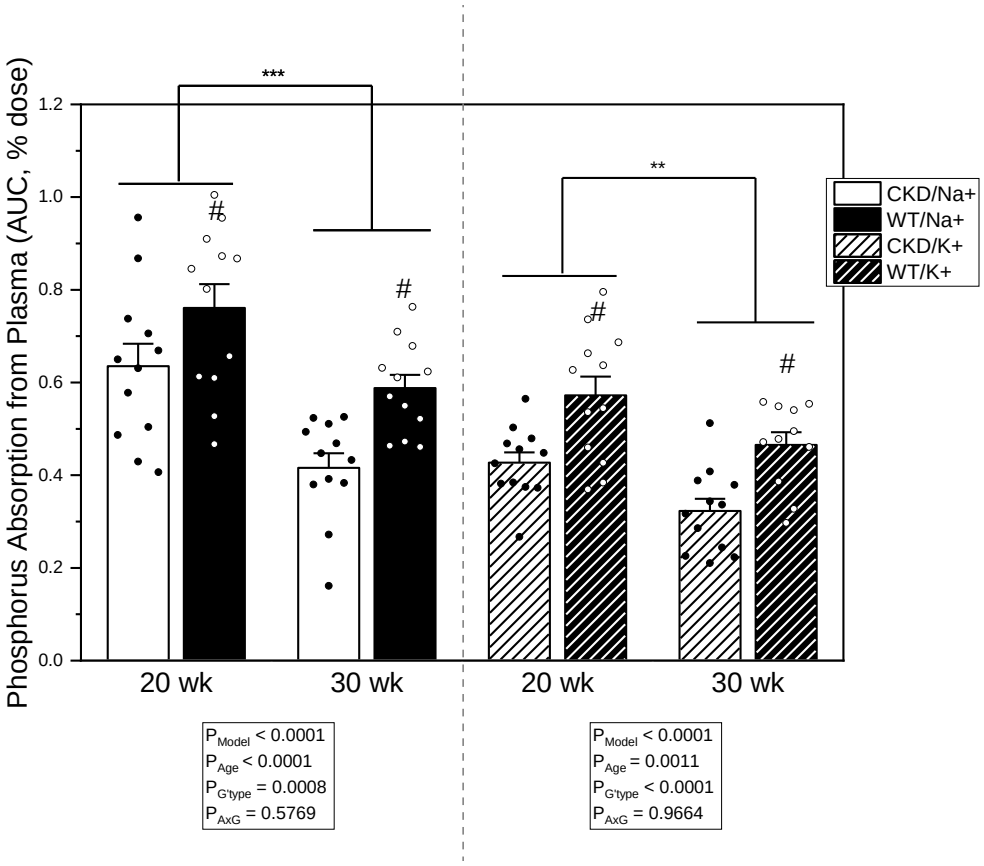

Figure 4.2. Jejunal phosphorus absorption in plasma over 30 minutes by age (20 or 30 weeks) and genotype (CKD or WT) with or without sodium in the absorption buffer. Phosphorus absorption efficiency was calculated as % of initial dose of ^{33}P. Means and standard error bars are shown for each group. For rats given the absorption buffer with sodium (left), there was a main effect for age where 20 week old rats had higher absorption efficiency compared to both 30 week olds, and CKD rats had higher balance compared to WT, with no significant age x diet interaction. For rats given the absorption buffer without sodium (right), 20 weeks had a higher absorption vs 30 weeks, and CKD rats were higher than WT. Absorption buffer with sodium: CKD rats are shown with black bars and white dots, WT rats are shown with white bars and black dots. Absorption buffer without sodium: CKD rats are shown with black bars with white hashes and white dots, WT rats are shown with white bars with black hashes and black dots. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown. ** $p < 0.01$, *** $p < 0.0001$, # $p < 0.01$.

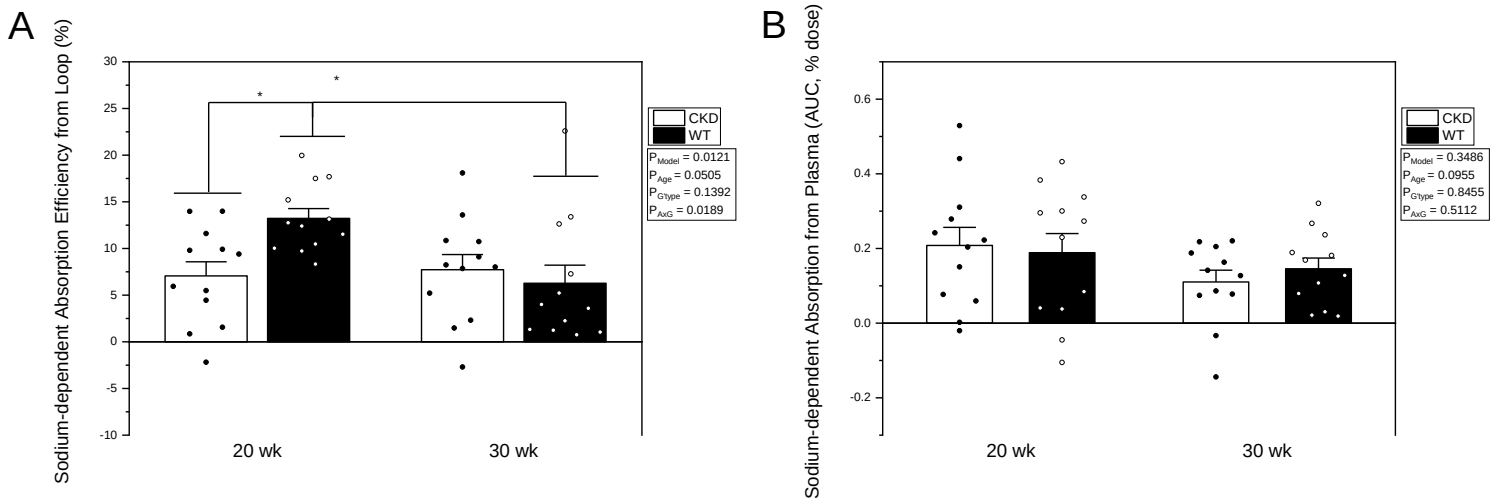

Figure 4.3. A) Sodium-dependent jejunal phosphorus absorption efficiency from loops by age (20 or 30 weeks) and genotype (CKD or WT). There was a significant age x genotype interaction, with 20 week CKD rats having higher sodium-dependent absorption efficiency compared to 20 week normal and 30 week CKD rats. B) Sodium-dependent jejunal phosphorus absorption in plasma over 30 minutes by age (20 or 30 weeks) and genotype (CKD or WT). The overall model was not significant. Means and standard error bars are shown for each group. Sodium-dependent values were calculated by result of each rats given the absorption buffer with sodium minus the average sodium-independent absorption for that genotype and age group. CKD rats are shown with black bars and white dots, WT rats are shown with white bars and black dots. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown. * $p < 0.05$.

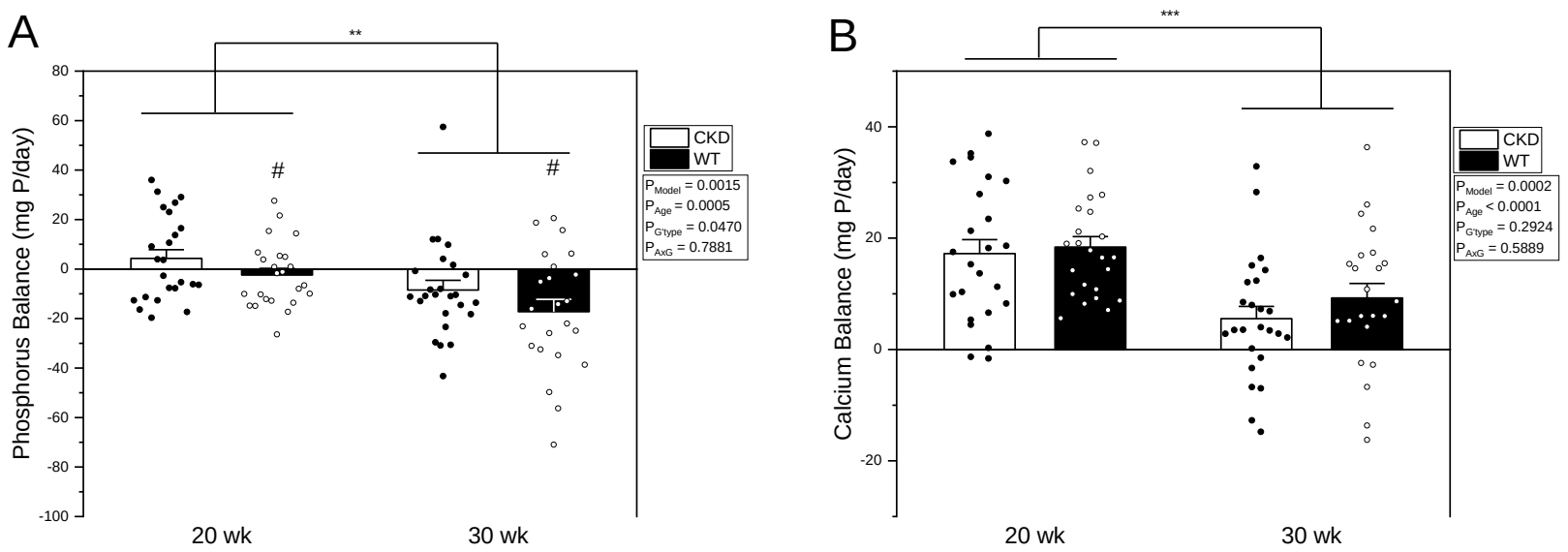

Figure 4.4. A) Phosphorus balance by age (20 or 30 weeks) and genotype (CKD or WT). There was a main effect for age where 20 week old rats had higher phosphorus balance compared to both 30 week olds, and CKD rats had higher balance compared to WT, with no significant age x diet interaction. B) Calcium balance by age and genotype. There was a main effect for age where 20 week old rats had higher calcium balance compared to 30 week olds, but there was no significant effect of genotype and no significant age x diet interaction. Balance for each mineral was calculated as intake – fecal + urine. Means and standard error bars are shown for each group. The CKD group is shown in black bars and white circles, and WT white bars with black circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown. ** $p < 0.01$, *** $p < 0.0001$, # $p < 0.05$.

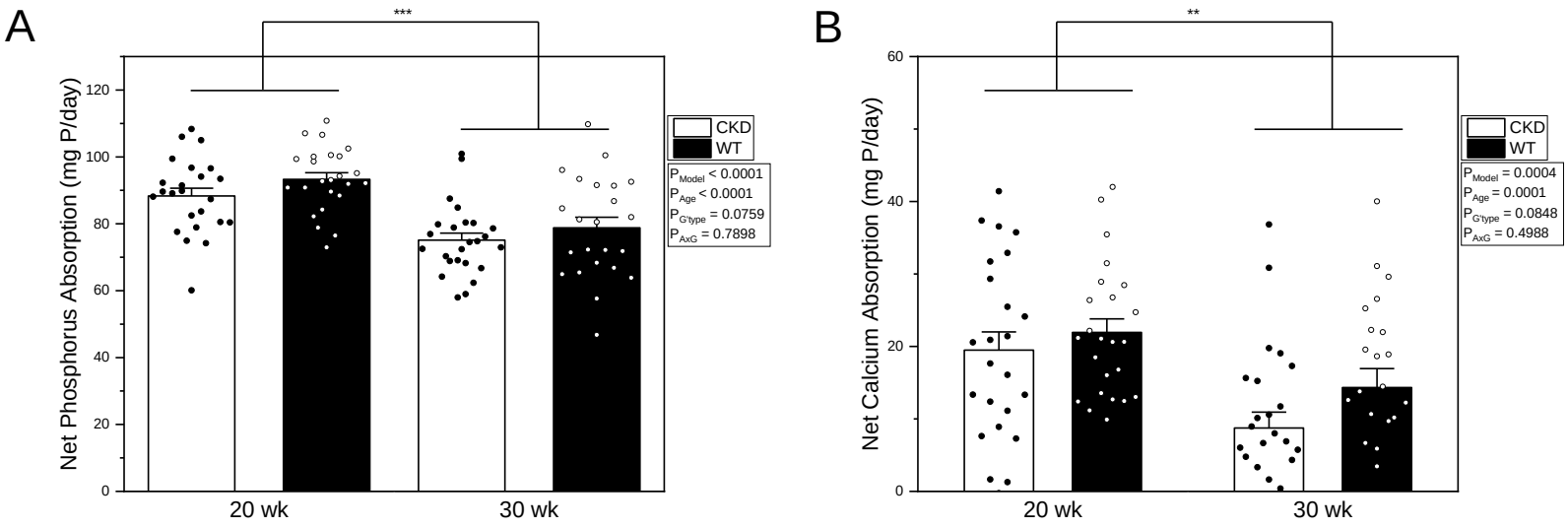

Figure 4.5. A) Net phosphorus absorption by age (20 or 30 weeks) and genotype (CKD or WT). There was a main effect for age where 20 week old rats had higher net phosphorus absorption compared to both 30 week olds, while there was no difference by genotype and no significant age x diet interaction. B) Net calcium absorption by age and genotype. There was a main effect for age where 20 week old rats had higher calcium balance compared to 30 week olds, with no difference by genotype and no significant age x diet interaction. Net absorption for each mineral was calculated as intake – fecal. Means and standard error bars are shown for each group. The CKD group is shown in black bars and white circles, and WT white bars with black circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of genotype ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown. ** $p < 0.01$, *** $p < 0.0001$.

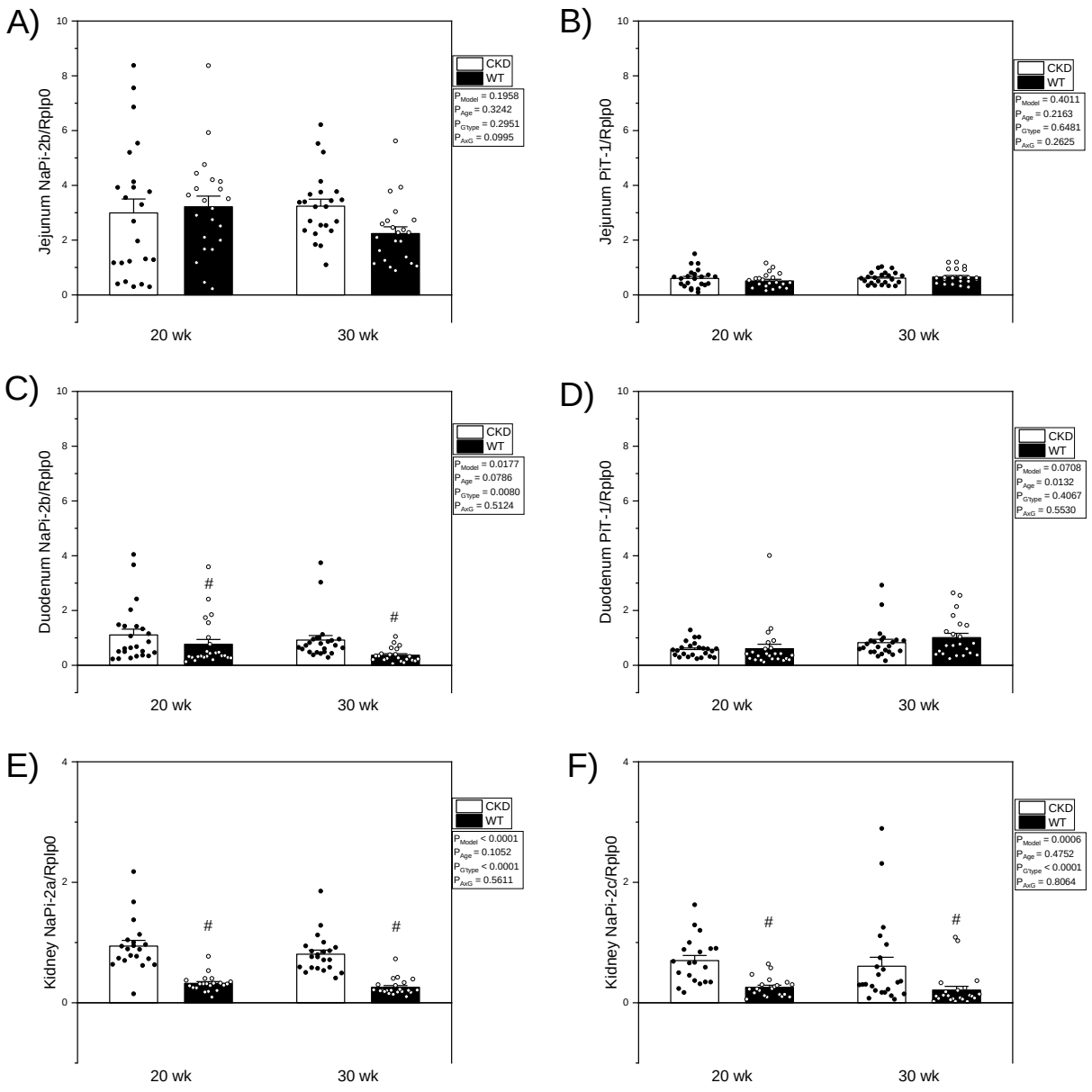

Figure 4.6. RNA expression of NaPi-2b, PiT-1 in jejunum and duodenum, and NaPi-2a and NaPi-2c in kidney. A) NaPi-2b mRNA was not different between groups in the jejunum. B) PiT-1 mRNA was not different between groups in the jejunum. C) NaPi-2b mRNA was lower in CKD rats vs WT in the duodenum. D) PiT-1 mRNA was not different between groups in the duodenum. E) NaPi-2a was lower in CKD rats vs WT in the kidney. F) NaPi-2c was lower in CKD rats vs WT in the kidney. Excluded outliers above 2 SD of the mean. The CKD group is shown in black bars and white circles, and WT white bars with black circles. ANOVA p-values for the overall model (P_{Model}), main effect of age (P_{Age}), main effect of diet ($P_{Genotype}$), and interaction of age and diet (P_{AxG}) are shown.

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CHAPTER 5: EFFECT OF ESTROGEN DEFICIENCY ON THE PROGRESSION OF CHRONIC KIDNEY DISEASE-MINERAL BONE DISORDER (CKD-MBD) IN FEMALE CY/+ RATS

Manuscript draft intended for submission to *Scientific Reports*

Abstract

Male Cy/+ rats develop progressive kidney disease and features of late stage chronic kidney disease-mineral and bone disorder by 35 to 38 weeks of age. However, female Cy/+ rats only begin to progress in disease severity after 40-44 weeks of age, which has precluded their use in studies to date. Animal and human reports suggest that estrogen may be protective against kidney decline. Therefore, we tested the hypothesis that estrogen deficiency would accelerate kidney disease in female Cy/+ rats. Eight female Cy/+ rats underwent ovariectomy (OVX) and eight underwent a sham surgery at 15 weeks of age. Blood was sampled every 5 weeks until 35 weeks of age, when the rats underwent a four-day metabolic balance, were sacrificed, and tibia collected for analysis. While OVX produced the expected changes in trabecular and cortical bone parameters, no difference between plasma blood urea nitrogen, creatinine, creatinine clearance, phosphorus, calcium, nor kidney weight were observed that would have indicated progression of kidney disease. These results indicate that estrogen deficiency does not produce a useful model of postmenopausal kidney disease in the female Cy/+ rat at these ages and duration post-ovariectomy. Development of other more feasible female models of progressive CKD are needed.

Introduction

CKD affects approximately 13.4% of adults worldwide (1), and prevalence and progression of the disease differ based on biological sex. Estimation of the global prevalence of CKD is higher in women than men (1), but a large cohort study showed a higher proportion of males than females at end stage renal disease (ESRD) (2). A meta-analysis of 68 studies on nondiabetic kidney disease concluded that kidney function declines slower in women than men (3). In concordance, ovariectomy (OVX) has been demonstrated to accelerate kidney disease in

various animal models (4-9), while exogenous estradiol administration attenuates the disease (5-12). However, some divergent studies have found no effect (13, 14) or even a protective effect (15, 16) of estrogen loss on kidney disease progression. In addition to the impact on the kidney, estrogen has well-established protective effects on bone (17). This is highly relevant as a common co-morbidity of CKD is CKD-mineral and bone disorder (CKD-MBD). CKD-MBD is characterized by biochemical abnormalities of mineral metabolism, bone disease, and vascular or other soft tissue calcification (18) that result in an increased risk for cardiovascular events, bone fractures, and death. Estrogen may modulate the main biochemical indicators of CKD-MBD, FGF-23, PTH, and 1,25D, either directly or indirectly (19). Thus, estrogen appears to be protective against kidney failure and associated co-morbid conditions. However, biological sex differences in the manifestation and progression of CKD-MBD through the stages of CKD are understudied.

The Cy/+ rat model of CKD is unique in that it has been characterized as a spontaneous slowly progressive model of CKD that exhibits all the key features of CKD-MBD and can be studied at earlier to later stages of disease progression (20, 21). The phenotype is the result of a missense mutation in *Anks6* that encodes for SamCystin, and results in renal cyst formation (22). Male Cy/+ rats experience a clear elevation in blood urea nitrogen (BUN) by 10 weeks of age (20) and show all features of late stage CKD-MBD by 35 to 38 weeks of age, including changes in additional plasma biochemistries such as creatinine, hematocrit, phosphorus and calcium, regulatory hormones PTH and FGF23, bone histomorphometric parameters, and vascular calcification (21, 23, 24). On the contrary, female Cy/+ rats do not experience an elevation in BUN comparable to 10-week-old males until 40-44 weeks of age (25, 26). This has resulted in minimal use of female Cy/+ rats in studies on CKD-MBD. Therefore, our primary aim of this study was to determine if reducing circulating estrogen (via OVX) would accelerate kidney functional decline in Cy/+ females compared to sham-operated females by 35 weeks of age. We hypothesized that OVX would accelerate kidney function decline and lead to CKD-MBD comparable to males by 35 weeks of age. Because most women with CKD are postmenopausal or amenorrhoeic due to the disease (27), this model would be translationally relevant to a large percentage of women with CKD who have concurrent estrogen-deficiency (or postmenopausal) osteoporosis.

Materials and Methods

Animals

Sixteen female Cy/+ rats were studied from our breeding colony at Purdue University. Heterozygosity for the Anks6 mutation was determined by ear punch and genotyping (Transnetyx, Memphis, TN). Rats were randomly assigned to shoe-box cages (2 rats per cage), and within each cage, randomly assigned to undergo OVX (N = 8) or sham (N = 8) surgery at 15 weeks of age (described below). Blood was drawn at 10, 20, 25, 30, and 35 weeks of age. Rats were fed standard rat chow containing 0.7% phosphorus and 1.0% Ca (Envigo Teklad 2018, Madison, WI) and water *ad libitum* until 24 weeks of age, at which time they were switched to an *ad libitum* casein-based diet (0.7% phosphorus and 0.7% calcium) which we have previously shown to lead to more consistent and accelerated kidney decline in Cy/+ males (TD.04539, Envigo Teklad, Madison, WI) (21). Animals were fed this diet until sacrifice at 35 weeks of age. At 13 days prior to sacrifice, rats were transferred to wire-bottom metabolic cages and a four-day phosphorus and calcium balance was performed from 9 to 5 days prior to sacrifice. Five days prior to sacrifice, rats were transferred back to shoe-box cages. Body weights were taken weekly. The light-dark cycle was maintained from 6:30AM-6:30PM. This protocol was approved by the Purdue University Animal Care and Use Committee.

OVX and Sham Procedures

Animals undergoing the OVX or sham surgery procedures were shaved on the dorsal midline. All appropriate steps were taken for an aseptic surgery. One incision ~2 cm was made on the dorsal midline. The skin was bluntly dissected from the abdominal wall to both sides. The incision was pulled to the left side and a small ~10 mm incision was made with scissors through the abdominal wall. The ovary was externalized with thumb forceps so that the ovary and the end of the uterine horn were exposed. A silk ligature was tied between the end of the uterine horn and the ovary. Another silk ligature was tied between the ovary and the ovarian artery. Two cuts were made to excise the ovary from the artery and the uterus. The abdominal wall was closed with an absorbable suture and then the skin was pulled to the opposite side to remove the other ovary by the same method. When both ovaries were removed, the skin was stapled shut. Buprenex was administered subcutaneously approximately 15 minutes before the end of the

surgery for pain management. Rats woke up on a towel and once sternal recumbency occurred they were moved back to their cages. The staple was removed 5-7 days post-surgery. Sham-operated rats underwent the same surgical procedure, excluding the ligation and removal of the ovaries.

Tissue Collection

At sacrifice, rats were anesthetized with isoflurane, the thoracic cavity was opened, and blood was collected from the vena cava resulting in death by exsanguination. Kidneys and uteri were excised and weighed. The left tibia was excised, cleaned of surrounding soft-tissue, and stored in 10% neutral buffered formalin for 3 days, then transfer to 70% ethanol and stored at -20°C until the time of microCT analysis.

Phosphorus and Calcium Balance and Percent Net Absorption

Over the four days of metabolic balance, all urine and feces were collected and diet weighed daily to assess 4-day average phosphorus and calcium balance and net absorption. Feces and diet were ashed in a muffle furnace (Thermolyne Sybron Type 30400, Dubuque, IA) for 10 days at 600ºC. Feces were then diluted 1400X and diet 60X with 2% nitric acid. Urine was diluted 11X with 2% nitric acid. Phosphorus and calcium in urine, feces, and diet were quantified by inductively coupled plasma-optical emission spectrophotometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT). Urine creatinine was determined by colorimetric method (QuantiChrom Creatinine Assay Kit; BioAssay Systems, Hayward, CA). Four-day phosphorus balance was calculated as dietary phosphorus intake (mg/d) minus urine and fecal phosphorus excretion (mg/d), and percent net phosphorus absorption as phosphorus intake (mg/d) minus fecal excretion (mg/d) / phosphorus intake (mg/d). Calcium balance and percent net calcium absorption were calculated similarly.

Plasma Biochemistries

Plasma stored at -80C was thawed and analyzed for phosphorus, calcium, blood urea nitrogen (BUN), and creatinine by colorimetric assay (Phosphorus Kit: Pointe Scientific Inc., Canton, MI; Calcium Kit: Pointe Scientific Inc., Canton, MI; BUN: QuantiChrom Urea Assay

Kit, BioAssay Systems, Hayward, CA; Creatinine: QuantiChrom Creatinine Assay Kit; BioAssay Systems, Hayward, CA).

microCT

Proximal tibia were analyzed by μCT (Skyscan 1172, 12μm resolution) using protocols similar to our previous studies (you can cite any of our CKD papers with CT). Trabecular microarchitecture was obtained from a 1mm region of interest selected approximately 1mm distal to the tibial growth plate. Bone parameters assessed included trabecular b one volume/tissue volume (BV/TV, %), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp). Cortical bone analysis was performed on a single slice located 1.5mm distal from the metaphysis region of analysis with outcome parameters including cortical bone area (Ct.Ar), and cortical thickness (Ct.Th). There was no cortical porosity noted in any of the animals (OVX or Sham).

Statistics

Repeated measures analysis of variance (ANOVA) for differences between groups was performed for all plasma biochemistries. Unpaired t-tests was performed for comparison of mean differences in uterine and kidney weights, mineral balance and net absorption, and bone outcomes between OVX and Sham. Statistical significance was set at $\alpha < 0.05$. Statistical Analysis Software version 9.4 (SAS Institute, Cary, NC) was used for all statistical analyses. Results are reported as mean ± SEM.

Results

OVX was deemed successful by the significantly lower uterine weight observed at 35 weeks in OVX rats compared with Sham (0.16 ± 0.01 g vs 0.78 ± 0.06 g, respectively; $p < 0.0001$), and greater increase in bodyweight after OVX surgery ($p < 0.0001$, **Figure 5.1**). Plasma creatinine ($p = 0.43$), BUN ($p = 0.09$), phosphorus ($p = 0.61$), and calcium ($p = 0.39$) were not different between OVX and Sham groups (**Figure 5.2**). Creatinine clearance was not different between groups (4.2 ± 0.2 mL/min vs 4.1 ± 0.2 mL/min for OVX and Sham respectively, $p = 0.83$). In addition, kidney weight was not different between groups (1.417 ± 0.075 g vs 1.382 ± 0.050 g, $p = 0.70$).

Phosphorus balance was lower in OVX rats compared to Sham (-2.8 ± 2.6 mg/day vs 5.4 ± 2.5 mg/day, $p = 0.04$), while calcium balance was also numerically lower but did not reach statistical significance (0.6 ± 2.9 mg/day vs 7.1 ± 2.8 mg/day, $p = 0.12$) (**Figure 5.3**). Similarly, percent net phosphorus absorption was numerically lower in OVX rats vs Sham, but did not reach statistical significance (43 ± 2 % vs 48 ± 2 %, $p = 0.08$), while percent net calcium absorption was numerically lower but not significant in OVX (5 ± 4 % vs 12 ± 3 %, $p = 0.12$) (**Figure 5.3**). Individual components of balance are listed in **Table 5.1**.

In the tibia, BV/TV and Tb.N were lower in OVX rats ($p < 0.0001$ for both), and Tb.Sp higher ($p < 0.0001$) vs Sham **(Table 5.2)**. Tb.Th was not different ($p = 0.15$) **(Table 5.2)**. No cortical porosity was noted for any rats.

Discussion

Although OVX of Cy/+ female rats produced the expected phenotypic changes in body and uterine weights, and trabecular bone volume, there was no indication of advancing kidney disease as measured by creatinine clearance, plasma creatinine, BUN, phosphorus, and calcium, nor kidney weight. Furthermore, there was no reduction in intestinal calcium absorption as is seen in early CKD due to reduced $1,25(OH)_2$-vitamin D. Finally, although OVX did produce changes in cancellous bone, there were no changes to cortical bone which is the primary pathogenesis observed in the Cy/+ male animals with uremia. These findings were contrary to our hypothesis that OVX would hasten kidney disease progression and the development of CKD-MBD in female Cy/+ rats.

The renoprotective potential of estrogen on kidney function has been examined in various animal models with conflicting results. Some studies have shown an increase in glomerulosclerosis in response to OVX. This has been demonstrated in sclerosis-prone ROP Os/+ mice (4), 5/6 nephrectomized Wistar rats (5), in rats with streptozotocin-induced diabetic nephropathy (6), Dahl salt-sensitive rats (7), and in female Imai rats that develop spontaneous hypercholesterolemia (9). Within these experiments, OVX also increased tubulointerstitial fibrosis (5, 7), proteinuria (5), and serum creatinine with a trend toward decreased creatinine clearance (7). Further, administration of estradiol tended to mitigate the changes, with improved glomeruloscerosis (5-7), tubulointerstitial fibrosis (5-7), proteinuria (5, 6), creatinine measures (5, 6), and TGF-ß expression (6). In female Imai rats, a protective or aggravative effect of

estradiol on glomerulosclerosis was dependent on dose (9) which may be mediated by growth hormone (28). Additionally, exogenous estradiol in spontaneously hypertensive rats that underwent uninephrectomy, or in female db/db mice, male Imai rats, or male Sprague-Dawley rats has been demonstrated to reduce age-related glomerulosclerosis (8, 10-12), tubulointerstitial fibrosis (8), as well as albuminuria (10) and proteinuria (12). The estradiol metabolite 2-hydroxyestradiol has also been shown to be renoprotective in puromycin-aminonucleoside model of nephropathy (29). Protective effects of estrogen on renal function have been suggested to be mediated by a reduction in extracellular matrix protein accumulation (30, 31).

In contrast, OVX did not worsen kidney disease in female Munich-Wistar rats that progressively develop glomerular injury with age nor 5/6 nephrectomized Wistar rats (13, 14). Further, some studies have even shown apparent benefits of OVX to renal outcomes: OVX in female spontaneous hypertensive stroke-prone rats increased survival and reduced renal vascular pathology compared to sham surgery, which was reversed with estradiol administration (15). Similarly, in context of hyperlipidemia in both analbuminemic and Zucker rats, OVX was protective against glomerulosclerosis, while exogenous estradiol worsened it (16, 32). A previous study of OVX was conducted in weanling Cy/+ female rats. These rats underwent OVX at 4 weeks of age and did not exhibit subsequent changes in kidney weight, volume density of renal cysts, or BUN by 10 weeks of age compared to sham rats, however testosterone administration induced cystic disease progression (33). Our findings confirm that this lack of effect of OVX on kidney disease progression is not limited to growing rats in the Cy/+ model of CKD.

Sex differences in the progression of kidney disease have been observed in both animals and humans. Previous studies have demonstrated that in the Cy/+ rat (Sprague Daley background), females do not develop the pronounced azotemia and fibrosis from cystic disease until much older ages compared with males (25, 26). Interestingly, castration slows progression of CKD in the Cy/+ male rats but the present study did not demonstrate acceleration with loss of estrogen in the female (34). Other models also have predominant CKD in male animals. Male Munich-Wistar hypertensive rats have elevated urinary protein excretion that increases with age that is not observed in females (35), and male Imai hypercholesterolemic rats develop nephritis whereas females do not (36). It has long been observed that nephrectomized male Wistar rats develop nephritis more severely than females (37). In humans, population studies show that end-

stage renal disease incidence is higher in men than premenopausal women, but sex differences begin to lessen around menopausal years (38). A meta-analysis of 68 studies on nondiabetic kidney disease progression concluded that kidney function in men declines faster than in women (3). In premenopausal women, bilateral oophorectomy at age ≤45 years was associated with an elevated risk of CKD as assessed by estimated glomerular filtration rate (39). However, a meta-analysis of hormone replacement studies in postmenopausal women found no significant effect on albuminuria or proteinuria when assessed together but in a subgroup analysis of studies assessing only albuminuria there was a small favorable effect of hormone replacement lowering albuminuria (40). Thus, the Cy/+ male rat is a more suitable model of progressive CKD and resulting CKD-MBD than the female, consistent with human studies.

Interestingly, in our study, OVX rats had lower, but not statistically significant, percent net phosphorus absorption that may suggest that estrogen influences intestinal phosphate transporters. There is some experimental evidence to support this notion. Acute 17ß-estradiol injection at 2 mg/kg body weight in rats increased intestinal brush boarder membrane vesicle uptake and the mRNA and protein expression of the main known intestinal phosphate transporter, sodium phosphate cotransporter 2b (41). However, in contrast, a study in female rats of similar age to those in ours found no change in net phosphorus absorption with injection of 5 or 40 ug/kg 17ß-estradiol for 21 days (42).

In summary, OVX did not produce an acceleration of kidney disease in the Cy/+ rat model of kidney disease. Our findings suggest that estrogen is not the protective factor in the disease progression in this animal model.

Table 5.1. Components of balance for phosphorus and calcium

Component	**Phosphorus**			**Calcium**		
	OVX	**Sham**	***P***	**OVX**	**Sham**	***P***
Balance (mg/d)	-2.8 (2.6)	5.4 (2.4)	0.04	0.6 (2.9)	7.1 (2.8)	0.12
Net absorption (mg/d)	55.1 (2.7)	69.5 (2.8)	0.002	4.6 (3.0)	12.7 (2.8)	0.07
Net absorption (%)	43.3 (2.2)	48.4 (1.6)	0.08	5.0 (3.3)	12.0 (3.6)	0.12
Fecal excretion (mg/d)	72.1 (3.3)	74.3 (2.6)	0.61	88.3 (6.6)	92.3 (2.9)	0.39
Urine excretion (mg/d)	57.8 (1.4)	64.2 (1.2)	0.005	4.0 (0.3)	5.6 (0.4)	0.009
Dietary intake (mg/d)	127.2 (2.1)	143.8 (3.1)	0.0005	92.8 (1.5)	105.0 (2.2)	0.0005

Values are mean ± SE.

Table 5.2. Microstructural parameters of cancellous bone of the tibia measured by micro-CT

	OVX (n = 8)	**Sham (n = 8)**	***P***
BV/TV (%)	0.99 ± 0.34	17.45 ± 1.59	< 0.0001
Tb.N (mm^{-1})	0.14 ± 0.04	2.16 ± 0.13	< 0.0001
Tb.Th (mm)	0.07 ± 0.008	0.08 ± 0.003	0.15
Tb.Sp (mm^{-3})	0.84 ± 0.02	0.27 ± 0.01	< 0.0001
B.Ar (mm^2)	5.93 ± 0.07	5.22 ± 0.07	< 0.0001
Cs.Th (mm)	0.48 ± 0.004	0.39 ± 0.005	< 0.0001

Values are mean ± SE. BV/TV (bone volume (BV)/Tissue volume (TV)); Tb.N (trabecular number); Tb.Th (trabecular thickness); Tb.Sp (trabecular separation); B.Ar (cortical bone area); Cs.Th (cortical thickness).

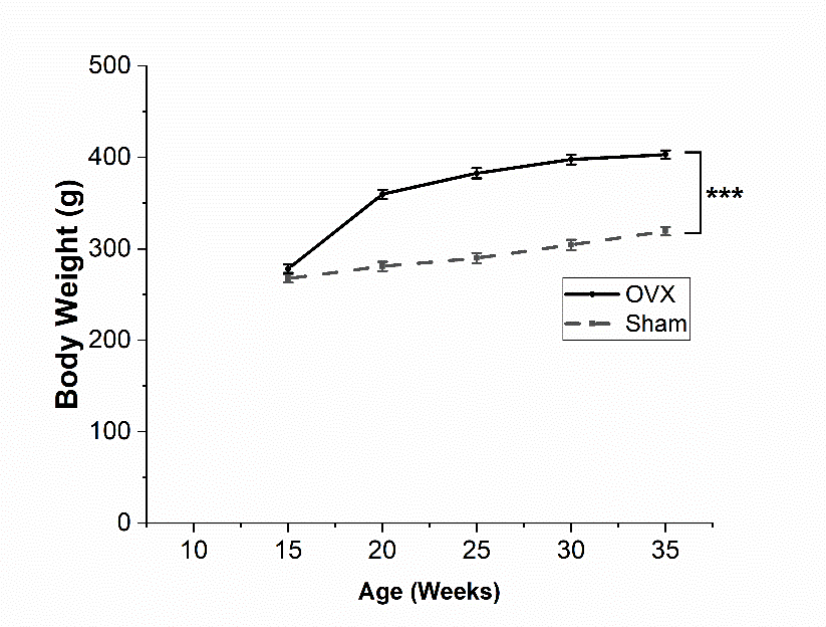

Figure 5.1: OVX resulted in higher body mass relative to Sham. Values presented are mean ± SE. *** p<0.0001 between groups.

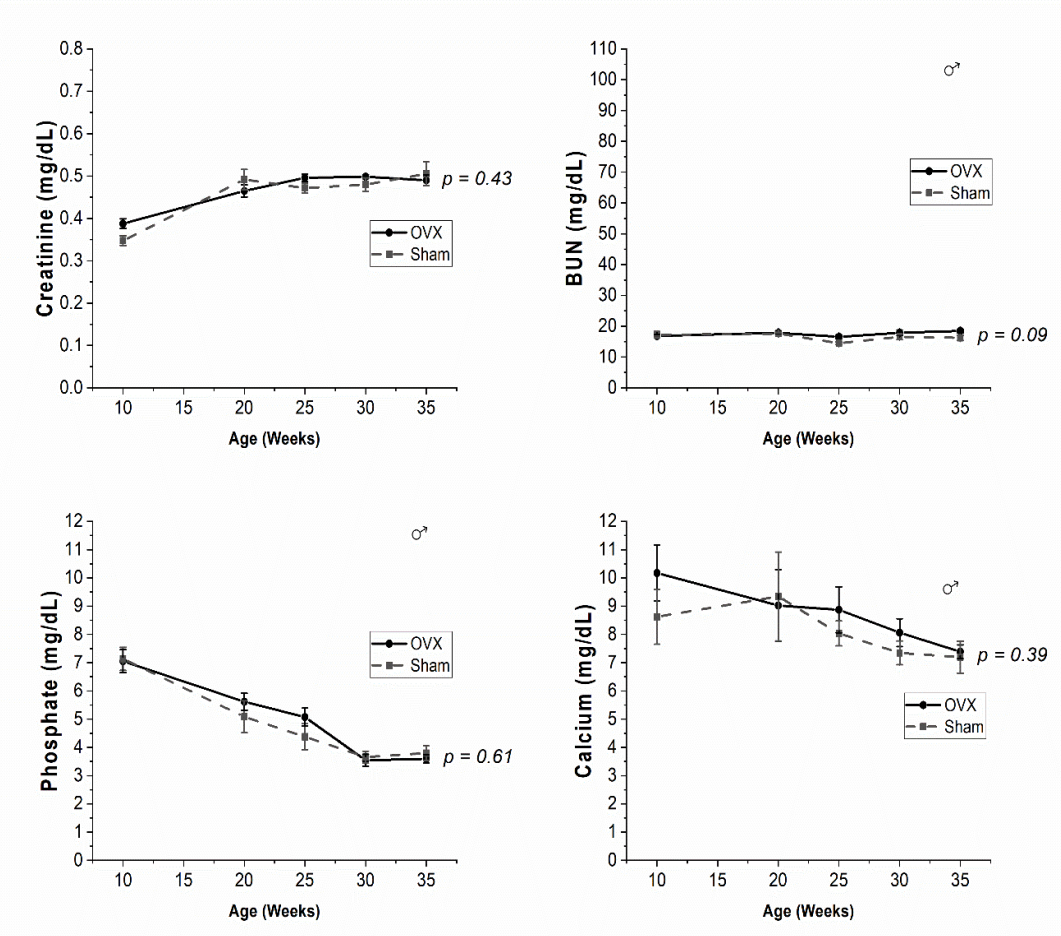

Figure 5.2: Plasma biochemistries over time between OVX and Sham surgery. Plasma creatinine, BUN, phosphate, and calcium were not different between the groups. ♂ signifies the mean of male Cy/+ rats at 34 weeks from Moe et al. 2009 for comparison (21). Values presented are mean ± SE.

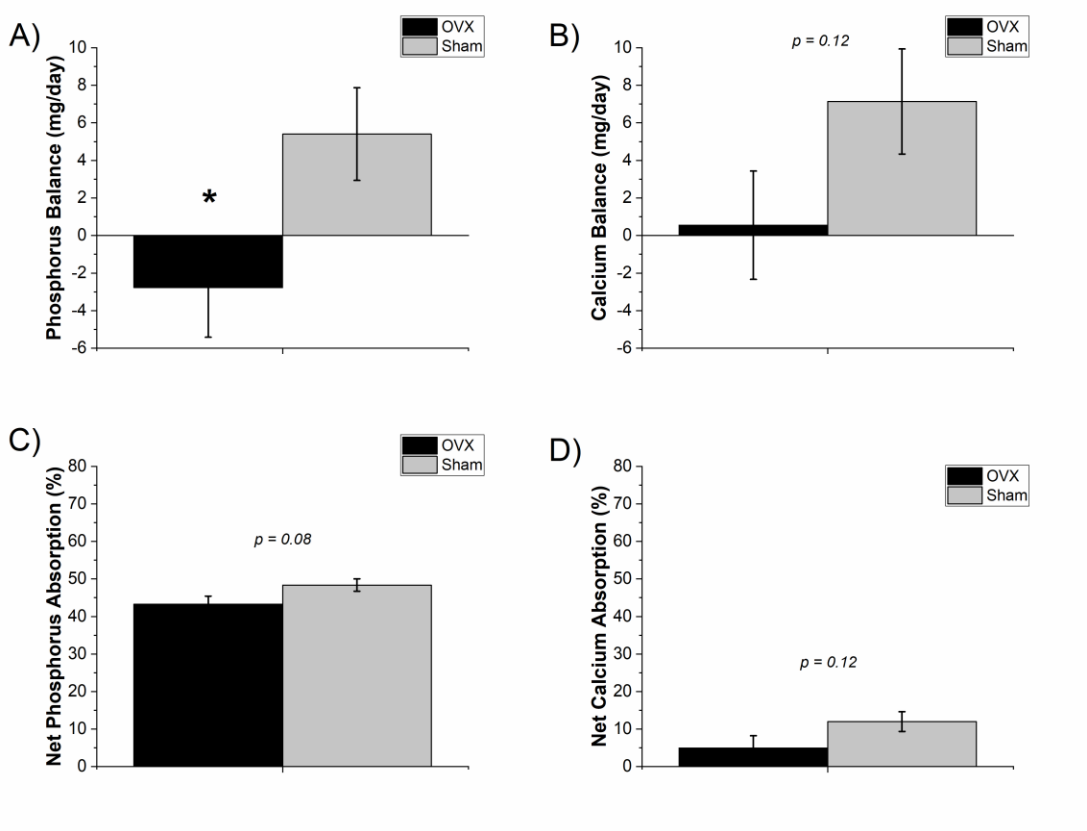

Figure 5.3: Mineral balance and net mineral absorption at 35 weeks by OVX or Sham surgery. A) phosphorus balance was lower in OVX rats vs Sham. B) calcium balance was not different between the groups. C) percent net phosphorus absorption and D) percent net calcium absorption were not different between groups. Values presented are mean ± SE. * $p<0.05$.

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CHAPTER 6: DISCUSSION

Summary & Synthesis

Phosphorus Balance in Adolescent Girls and the Effects of Dietary Calcium

In adolescent females, increasing calcium intake from ~800 mg/day to ~1400 mg/day through supplementation decreased urinary excretion of phosphorus, suggestive of a renal adaptation in response to a reduction in phosphorus absorption. However, the decrease in net absorption was too small to be detected in fecal phosphorus, nor overall balance, which was positive but not different between the calcium group and placebo. Like previous studies, we also showed a large underestimation of dietary phosphorus from a nutrient database compared to measured values. This secondary analysis suggests that changes in calcium intake within normal to just above recommended values in adolescents does not bind enough phosphorus to warrant concern toward mineral balance.

Rat Phosphorus Absorption *In Situ* Studies

Our two rat studies in healthy Sprague Dawley male rats and Cy/+ male rats together suggest that factors such as the level of phosphorus in the diet, age, and kidney disease progression are less impactful on jejunal intestinal phosphorus absorption efficiency adaptation than in vitro and ex vivo methods of measuring uptake suggest. We found no change in absorption efficiency by disappearance from the ligated loop or appearance in plasma in response to manipulating the level of dietary phosphorus in healthy Sprague Dawley males. There was a small elevation in younger (10 week) rats versus 20 and 30 weeks. Gene expression of NaPi-2b in the jejunum supported these trends. Further, net absorption of phosphorus from metabolic balance also showed an elevation at 10 weeks of age. Additionally, the net absorption results reflect that passive absorption drives much of the overall phosphorus absorption, with a stepwise increase in phosphorus absorption with the amount of phosphorus in the diet. In our CKD rats, surprisingly, CKD *increased* intestinal absorption efficiency assessed by both disappearance from loop and absorption into plasma, albeit by a small amount, contrary to the

hypothesized decrease based on *in vitro* studies. However, net phosphorus absorption results from metabolic balance support a small increase as well. And, these results were actually consistent with the one other previous rat study of CKD (5/6th nephrectomy) rats versus normal using the *in situ* ligated loop test (1). Unexpectedly, these rats at 20 weeks of age experienced higher absorption efficiency versus 30 weeks, contrary to the healthy Sprague Dawley rats. This was also consistent with the net phosphorus absorption data. Absorption findings for the age effects in both of these rat studies corresponded to the 1,25D results. Given its known role in stimulating intestinal phosphorus absorption, it may play a role in strain differences of phosphorus absorption decline with age. Interestingly, the effect of CKD on phosphorus absorption was divorced from its effect on 1,25D levels, consistent with the findings of Marks et al. (1). The lack of significant adaptive capacity of intestinal phosphorus absorption efficiency in response to these factors (dietary phosphorus level, age, and kidney function) question the physiological relevance of these adaptations that have been observed in prior *in vitro* and *ex vivo* studies. Also, because of the high affinity to phosphate of NaPi-2b compared to physiological luminal phosphate concentrations (2), it is unlikely that experiments done *in vitro* under conditions optimized to see changes in active uptake via NaPi-2b will reflect what occurs *in vivo*.

Effects of Ovariectomy in Cy/+ Females on the CKD Phenotype

The inability to use female Cy/+ rats because of the very delayed onset of CKD at much older ages compared with males is problematic for exploring sex-related differences in intestinal phosphorus absorption using this progressive disease model. Our finding that ovariectomy at 15 weeks of age did not accelerate CKD progression by any measure – BUN, creatinine, creatinine clearance, nor plasma phosphorus or calcium, nor kidney weight – indicates that it cannot be a useful model for progressive CKD, supporting previous work of ovariectomy at 4 weeks of age, observing markers to 10 weeks in Cy/+ females (3). Other models could be used to study CKD pathophysiology and sex differences in both males and females, such as the 5/6th nephrectomy model or the adenine-induced CKD models. The adenine-induced model may be particularly useful because both mild and severe levels of disease can be achieved based on duration of adenine administration (4). Female animal model studies in CKD are sparse in general, and to our knowledge none exist in regard to studying intestinal phosphorus absorption (5, 6).

Strengths and Limitations

Our studies have several strengths and limitations that are discussed within the individual chapters 2-5. Some of these will be highlighted here. First, our metabolic balance studies in both adolescent girls and rat models had dietary intakes and environmental conditions that were tightly controlled, and measures were meticulously timed. As such, our carefully conducted balance studies in each of these experiments are strong complements to the radiolabeled phosphorus absorption measures by in situ ligated loop. Of course, with more control over experimental conditions, the generalizability and relevance to more diverse, complex, and free-living populations is unclear. Our choice in using rats as an animal model is a strength because humans and rats have similar patterns of intestinal phosphorus absorption in regard to the intestinal segments (which is not the case for mice and humans) (Chapter 1, Table 2). However, unpublished reports on intestinal phosphate gene expression between species is not entirely consistent. NaPi-2b expression was measured in a stepwise decrease from duodenum to jejunum and ileum in rats, which matches humans (7). A separate report found that NaPi-2b expression was higher in the jejunum than duodenum in rats, but this was reversed in humans (8). PiT1 expression patterns paralleled NaPi-2b in each species (7, 8). These conflicting results are however reflected in rat studies that have found a higher absorption rate in the duodenum (9) and jejunum (10) by the same group. There is limited comparison in humans, although the jejunal phosphorus absorption has been reported to be higher than in the duodenum (11). The capacity for adaptation in human intestinal segments has not yet been quantified.

The ligated loop method has been shown to be sensitive to reflect large changes in calcium absorption in response to calcium restriction and aging in mice (12), and as such we expected it to reflect those changes phosphorus absorption as well. Indeed, the ligated loop has been used in rats to show changes phosphorus absorption in response to glucocorticoids (13), and some (inconsistent) changes in response to dietary restriction of phosphorus have been observed (5). Both of these previous studies selected the duodenum to study effects of their interventions. Although the duodenum in rats has significant active absorption of phosphorus (14), and compartmental analyses of intestinal phosphorus absorption indicate significant absorption in the distal small intestine due to long length and residence time (15, 16), we selected the jejunum for the following reasons: First, NaPi-2b is highest expressed in the jejunum in the rat (14). Second, previous evidence suggests that the adaptive response in NaPi-2b mRNA and protein as well as

phosphate BBMV uptake to chronic dietary phosphorus restriction occurs in the jejunum but not duodenum in rats (17, 18). Thus, the lack of change in response to dietary restriction, and the small change in response to CKD progression and age, suggests that whole intestinal changes in phosphorus absorption are minimal.

While *in vitro* methods of measuring uptake suggest that sodium-dependent transport predominates at low (i.e. 0.1 mM) phosphate concentration, it is difficult to estimate this when utilizing the ligated loop and oral gavage, which have produced estimates that the bulk of absorption occurs via the sodium-independent component (Chapter 1, Table 1). However, part of this reason may be because of endogenous sodium secretion into the lumen during the *in situ* and *in vivo* absorption tests producing a higher luminal phosphate concentration that favors sodium-independent absorption (10). The wide variation in measuring luminal phosphate indicates that it is difficult to estimate how much sodium-independent absorption this could drive (10, 19). Still, Marks et al. estimated what should be interpreted as the minimum sodium-dependent absorption in the rat jejunum at about 32%, with a measured ~5.7 mM luminal phosphate (10), suggesting that it is a large enough component that changes should still be observable in response to interventions. Although we attempted to measure sodium-independent absorption with the ligated loop in our study in CKD rats, it is challenging to conceptualize how much sodium-dependent absorption this measure actually contains when measured in situ due to potential for endogenous secretion. In our Sprague Dawley study of effects of age and dietary phosphorus level, we did not attempt to measure sodium-independent absorption because others have shown this to be load-dependent, and unregulated (20-23). However, this would have been interesting to measure for age effects given the report of a change in BBMV uptake with age (24), and thus we are limited in not being able to compare this to the ligated loop method. It is notable that the ligated loop provides similar estimates of relative absorption rates compared to the everted sleeve, indicating that it consistent to *in vitro* techniques in this regard (10). We believe *in situ* and *in vivo* methods are important to utilize to characterize the importance of changes in absorption, and thus are a major strength of our work. Net absorption measures from metabolic balance reinforced our conclusions for both experiments utilizing the ligated loop. As pointed out by Marks et al. (10), other examples in biology exist to support this sentiment: e.g. GLUT2 was observed to rapidly internalize away from the brush border membrane during in vitro preparation of intestinal tissue, and thus was not recognized until later.

Each of our studies is somewhat limited in sex related comparisons. The inclusion of only adolescent females in the Camp Calcium secondary analysis was limited to its design. The healthy rat study manipulating dietary phosphorus and age was only done on males to maintain homogeneity in an already large study, however given some changes observed in response to dietary restriction of phosphorus in females with the ligated loop (5), it is important for future work to compare sex differences. The Cy/+ female rats do not develop progressive kidney disease as observed in males, as we showed even after ovariectomy. Thus, we are limited in using this model to study sex differences in absorption. Other models such as the 5/6 nephrectomy or adenine induced CKD could be used for these questions.

We assessed gene expression of NaPi-2b and PiT-1 in the intestines in our rat absorption studies, but not PiT-2. We focused primarily on NaPi-2b expression since this transporter has been estimated to account for 90% of sodium-dependent uptake by Sabbagh and colleagues (25), and added measurement of PiT-1 expression. PiT-2 expression in all intestinal segments in the rat is minimal (17), and mRNA was unchanged in response to dietary phosphorus restriction (21). A major limitation is the lack of data on protein expression levels for each of these transporters, particularly given that there is discordance in whether NaPi-2b is post transcriptionally regulated in response to dietary restriction of phosphorus (Chapter 3), and there is limited data on protein expression levels in CKD (Chapter 4).

Finally, the Cy/+ rat model is a well-documented model of autosomal dominant polycystic kidney disease (ADPKD) (Chapter 5). However, ADPKD is the cause of only ~7-10% cases of hemodialysis (26), and thus results using this rat may not generalize to all etiologies of CKD, of which diabetes and hypertension are likely the primary (27). Further, the mutation in the gene Anks6, which is responsible for ADPKD in the Cy/+ rat, is thought to be responsible for a small proportion of ADPKD in humans (28), whereas mutations in PKD1 and PKD2 cause the majority of cases (26).

Future Directions

The translation of basic findings to clinical relevance is always challenging and tempered by additional considerations. Our results indicate a need for additional studies using physiological methods of assessing absorption (e.g. *in situ* ligated loop or *in vivo* oral gavage) to verify our findings. Recently, Turner et al. showed no difference in phosphorus absorption in

adenine-induced CKD animals after oral gavage of phosphorus (4). Marks et al. previously found no difference in 5/6 nephrectomized CKD animals with the ligated loop (1). Our results extend these findings to a genetic model of CKD, finding only a small increase with CKD. In response to dietary phosphorus restriction, absorptive adaptations have not yet been tested with the oral gavage method. Further, studies should assess both males and females within a cohort to observe if any sex differences exist, given the different results observed in females (5) and the effect of estrogen on transporter expression (29). Although it was recently confirmed that CKD rats still upregulate intestinal transporter expression in response to dietary phosphate restriction in the jejunum (18), it should be tested whether absorption increases to any significant degree. Further, Giral et al. (17) reported an intriguing overshoot phenomenon of phosphorus absorption when normal rats are restricted to consuming phosphorus for 4 hours each day and are acutely switched from phosphorus restriction to a high phosphorus diet. This phenomenon is important to explore further with physiological tests and in CKD models, as this may have relevance to certain human eating patterns such as intermittent fasting or dietary noncompliance in patients with CKD prescribed low phosphorus diets. Absorption tests examining the adaptive capacity of the intestine in response to dietary restriction of phosphorus in humans should explore whether the active transport of phosphorus is relevant to patient populations.

It remains unclear whether the differences observed between the ligated loop and *in vitro/ex vivo* methods reflect differences in residual phosphorus concentration in the loop, creating an environment minimizing active transport of phosphorus, or other factors. Methods are needed to quantify actual sodium-dependency of transport under different phosphate concentrations with the loop using positive controls with NaPi-2b inhibitors, or sodium binders to negate sodium-dependent transport when an absorption buffer without sodium is administered. Of various factors that have been shown to influence intestinal phosphorus absorption, e.g. dietary restriction of phosphorus, age, 1,25D, glucocorticoids, estrogen, energy status, etc. could be comparatively tested for their relative importance via different methods of uptake/absorption.

Finally, a priority should be placed on large randomized trials exploring the effect of restricting phosphorus absorption on clinical outcomes. While a moderate level of evidence exists on restricting dietary phosphorus to lower serum phosphate (30), limited placebo controlled trials evaluating these strategies on hard outcomes such as mortality exist (31),

precluding confidence in the relevance of understanding intestinal adaptations for patient outcomes.

Conclusions

The capacity of the intestine to adapt to regulate intestinal phosphorus absorption efficiency may be limited at the physiological level. Although we did observe differences in absorption efficiency in younger rats and in CKD rats, they were modest compared to existing *in vitro* experiments, despite a similar concentration of phosphorus in the absorption buffer used in our tests. The relevance of sodium-dependent transport in physiological conditions has been increasingly questioned (2), and our results are consistent with these reports. Importantly, our results suggest that an upregulation of phosphorus absorption efficiency in response to dietary phosphorus restriction may not be a concern for patient populations who are attempting to restrict phosphorus pharmacologically or through diet. In addition, there doesn't appear to be a downregulation of absorption in CKD, which would suggest that this pathway remains a contributor to absorption and may be appropriate to target pharmacologically. Finally, renal adaptation is sufficient to maintain positive phosphate retention in response to increasing dietary calcium from the average of ~800 mg in this age group to up to ~1400 mg/day in adolescent females, which allays concern that supplementation or dietary intake of calcium to just above recommendations may interfere with the increased phosphorus requirement in adolescents. Additional translational research is needed to further refine whether the adaptive capacity of the intestine is relevant toward disease outcomes.

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APPENDIX A. REVIEW OF PHOSPHORUS BINDERS AND OTHER AGENTS USED TO LIMIT DIETARY PHOSPHROUS ABSORPTION

Calcium-based binders

Several phosphate binders are currently in use in CKD, with calcium-based ones being the most commonly used, and calcium carbonate and calcium acetate the most common salts. Calcium-based binders were first used in the early 1990's with the theory that because calcium absorption decreased in CKD when serum 1,25 D levels fall, that the extra calcium will overcome the loss of active, vitamin D-mediated Ca absorption. Improved total calcium absorption would suppress secondary hyperparathyroidism, and the increased calcium would go to bone. In addition, calcium-based binders were proposed as a safer alternative than aluminum-based binders. Binding efficacy of calcium salts, as estimated by stool analysis, is approximately 45 mg of phosphorus per gram of calcium carbonate and calcium acetate in healthy humans. In dialysis patients, calcium carbonate binding was measured as between 15-19 mg phosphorus per gram, while calcium acetate was 27 mg per gram (1). Although the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines recommend limiting total calcium (including the binder dose) to 2 grams per day (2), in practice titration protocols using much higher doses are often required to control serum phosphate.

Many balance studies in healthy participants have examined the impact of dietary or supplemental calcium on phosphorus absorption and retention, on young women (3, 4), and men (5-7) , generally reporting a reduced urine phosphate output and increase in fecal phosphorus, so retention is unchanged, except for one study males and females which found a decrease in retention when increasing calcium from 202 mg to 1522 mg (8).

In populations with renal disease, calcium has been studied at KDOQI recommended levels and also at higher doses. For example, in 8 CKD patients consuming a placebo with a base diet of 957 mg calcium carbonate for a total of 2457 mg calcium, urinary phosphate was lower on the higher calcium diet, but net absorption, retention, and fasting serum phosphate were unchanged (9). PTH and FGF-23 were unchanged by the added calcium. However, when calcium-based binders are used in a titration protocol at high doses, they may effectively control

serum phosphate. Bleyer et al. started 83 hemodialysis patients on 667-2000 mg of calcium acetate 3 times per day, which could be increased every 2 weeks to maintain serum phosphate control (10). With this regimen, serum phosphate was successfully maintained in the range of 2.5 to 5.5 mg/dL for 8 weeks, whereas it increased without the binder. Thus, calcium can be an effective binder at high doses, and this suggests that blocking intestinal phosphorus absorption can effectively manage serum phosphate. However, the weight of the evidence from healthy populations and in CKD suggest that calcium-based binders are not efficacious for modifying phosphate retention at totals below ~2700 mg per day. This indicates that the KDIGO recommendation of at most two grams of calcium per day is insufficient to affect phosphorus balance.

Non-metallic binders

The development of non-metallic, non-absorbed binders has yielded two FDA approved drugs for the treatment of CKD: sevelamer-based and lanthanum-based products. Sevelamer carbonate binds phosphorus at 26 mg per gram, while lanthanum has a higher binding capacity of 135 mg per gram. The typical dosing schedule for sevelamer carbonate is ~800 mg to ~15 grams, while it is ~800 mg to ~3 grams for lanthanum carbonate (1).

Sevelamer-based and lanthanum-based drugs have been used in a number of trials to assess their ability to alter phosphorus balance and serum phosphate. In healthy participants and patients with renal disease, both sevelamer (10-13) and lanthanum (14-16) have been demonstrated to reduce absorption when the dose is titrated to serum phosphate concentration, but not when given as a fixed dose (17).

Ferric-based binders

Ferric citrate was FDA approved in 2014 as a CKD treatment. Balance studies in rats suggest that it binds 85-181 mg of phosphate per gram of elemental iron. Ferric citrate dissociates in the bowel, releasing ferric ion which precipitates with dietary phosphorus. Doses typically range from 1 to 12 grams per day, with each 1 gram tablet containing 210 mg ferric ion (18). The efficacy of ferric citrate on serum phosphate has been evaluated in humans, showing a reduced absorption (19, 20).

Cardiovascular outcomes from phosphorus binders

Because phosphate binders can effectively control serum phosphate, cardiovascular disease outcomes should be attenuated. However, there are concerns, particularly about calcium-

based binders on vascular calcification. In a recent balance and kinetics study in CKD patients, calcium carbonate increased bone calcium balance, but less than the overall increase in calcium balance, suggesting soft-tissue deposition (9). Indeed, several trials have directly compared calcium and sevelamer binders on cardiovascular outcomes and suggested that calcium increases risk for valvular calcification (21), artery and aortic calcification (22), and coronary artery calcium score (23). In a multicenter randomized controlled trial with 1068 hemodialysis patients, there was no difference in overall mortality from calcium-based binders vs sevelamer, however in the subgroup over 65, the mortality on sevelamer was lower compared to calcium (24). Currently there are no long-term studies on cardiovascular outcomes on lanthanum, but there is some concern about organ accumulation and toxicity (25). Ferric citrate increases serum ferritin and transferrin saturation, though it reduced the occurrence of serious adverse events compared to active control, suggesting that iron overload isn't a concern (20). Recently, a 9 month placebo-controlled trial of 148 patients in CKD stages 3-4 comparing calcium acetate, sevelamer carbonate, and lanthanum carbonate demonstrated an increase in calcification of the coronary arteries and abdominal aorta compared to placebo, even though serum phosphate was reduced by all treatments (26). There is an urgent need for follow-up placebo-controlled comparison studies assessing hard cardiovascular endpoints and/or mortality. Such studies can provide additional confidence whether reduction in intestinal absorption of phosphorus improves outcomes in kidney disease patients.

Additional challenges with phosphorus binders

In addition to cardiovascular concerns, there are several practical and physiological challenges to the binder approach to managing serum phosphate. They tend to be costly- for example, in 2002 sevelamer was about $4400 per year (27). There is also a large pill burden because of the doses required of each binder, and thus compliance is low. About ½ of hemodialysis patients do not comply with phosphate-binding medications (28).

Reducing dietary phosphorus improves renal function and mortality in animal models. Because of the difficulty in achieving this with a modern food supply, phosphorus binders are used to prevent intestinal absorption of phosphorus. Calcium-based, sevelamer-based, lanthanum-based, and ferric citrate binders may help control serum phosphate in renal disease when there are no restrictions on dose. However, calcium-based binders consistently demonstrate worse cardiovascular outcomes compared to sevelamer-based binders. In addition, a study

comparing 3 binders indicated that they all increased arterial calcification. Because binders reduce phosphorus dose, it is likely that there is an upregulation of active transport through NaPi-2b. Particularly because compliance is poor, missing a dose with a meal may result in a higher absorption of phosphorus than normal. Giral and colleagues maintained Sprague-Dawley rats on a low (0.1%) phosphorus diet for 7 days, then increased phosphorus to 1.2% for 1 day (29). Serum phosphorus was much greater (~17 mg/dL) after 4 hours compared to a group consuming the 1.2% phosphorus diet for all 7 days (~8 mg/dL). This rapid increase corresponded with an increase NaPi-2b protein in duodenal BBMV, suggesting that the intestine can rapidly adapt and compensate for prolonged phosphorus restriction. Interventions that reduce NaPi-2b expression could mitigate this problem and provide an alternative or complementary approach to phosphorus binders.

Additional Factors Known to Influence Intestinal P Absorption Efficiency

Agents targeting transport

Nicotinamide, the amide of nicotinic acid, is a compound that has been demonstrated to reduce phosphorus transport and NaPi-2b. It was first shown by Kempson et al to reduce renal phosphate transport as measured by rapid filtration, and increase urinary phosphate with an acute intraperitoneal injection in Sprague-Dawley rats. Because nicotinamide is a component of NAD, it was also tested on phosphorus transport. Indeed, addition of NAD to cultured renal BBMV decreased sodium-dependent phosphorus uptake (30). Subsequently, nicotinamide was demonstrated to reduce sodium-dependent uptake in rat intestinal jejunal BBMVs along with serum phosphate. Nicotinamide reduced NaPi-2b mRNA but not PiT1, PiT2, BNPI, or PiUS. In addition, serum 1,25D and VDR mRNA were reduced (31). Similarly, intestinal phosphate transport is decreased by nicotinamide injection in a rat model of chronic renal failure (5/6 nephrectomy), and this also corresponded to a decrease in NaPi-2b mRNA in the jejunum. Serum phosphate was reduced without a change in urinary excretion, and the progression of renal insufficiency was slowed, as measured by BUN and serum creatinine (32). Further, nicotinamide was shown to prevent an increase in serum phosphate by ~50% in response to a phosphate bolus in wild type mice but not in NaPi-2b knockout mice (33). Finally, clinical trials have shown that nicotinamide consistently reduces serum phosphate in humans in dialysis patients (34). Together, these studies indicate that nicotinamide reduces intestinal phosphorus transport and induces a

decrease in NaPi-2b expression. Further research is necessary to determine whether it acts through an increase in NAD, modulation of 1,25D/VDR, directly, or another mechanism.

NaPi-2b inhibitors are currently in development, although the limited published human data suggest that treatment of end stage renal disease patients for 2 weeks with the drug ASP3325 is insufficient to reduce serum phosphate (35).

Sodium/hydrogen exchanger isoform 3 (NHE3) inhibitors represent a novel method of inhibiting intestinal uptake. Several oral NHE3 inhibitors (tenapanor, NTX792, and NTX3572) were tested in healthy rats by Labonté and colleagues (36). NTX792 and NTX3572 reduced phosphorus uptake and urinary phosphorus, while tenapanor and NTX3572 increased fecal phosphorus in balance studies. In 5/6 nephrectomized Sprague-Dawley rats, tenapanor reduced vascular calcification in the aorta and stomach, and mitigated the decline in renal function. Serum phosphate and FGF-23 were also reduced. Interestingly, jejunal BBM NHE3 and NaPi-2b expression weren't changed, and NaPi-2b nor PiT1 were altered in a cell-based assay. Recently, King et al. further investigated the mechanism of tenapanor, finding that it reduces paracellular phosphate permeability by a reduced transepithelial electrical resistance, likely through a decrease in pH (37). Molecular identification of tight junction proteins that interact with phosphate remain elusive. Several phase I and II trials of tenapanor have been published that demonstrate reductions in serum phosphate from the drug (34).

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APPENDIX B. REVIEW OF THE ROLE OF PHOSPHATE IN SKELETAL MINERALIZATION

The majority of phosphorus in the body is contained within bone as hydroxyapatite. The homeostatic mechanisms controlling the deposition and release of phosphorus from bone are part of the axis discussed earlier. Hydroxyapatite is formed by osteoblasts, which are derived from mesenchymal progenitors. Various transcription factors control the differentiation of mesenchymal stems cells to osteoblasts. The secretion of type I collagen and other proteins form osteoid, which upon hydroxyapatite is deposited. Mineralization is regulated by the ratio of intracellular inorganic pyrophosphate and extracellular inorganic phosphate in osteocytes; with an excess of phosphate, mineralization proceeds and excess of pyrophosphate inhibits. The level of phosphate is controlled by the amount in diet or renal reabsorption, or by a number of positive regulators such as PHEX, DMP1, and negative regulators such as FGF-23. In addition, alkaline phosphatase can synthesize it from pyrophosphate (1).

High serum and dietary phosphate has been shown to increase FGF-23 secretion (2), and also PTH primarily because of a reduction in plasma ionized calcium (3). These hormones also begin to elevate in the early stages of CKD-MBD (4), serving to regulate serum phosphate concentration. However, also have secondary actions on bone mineralization. Nearly 100% of long-term dialysis patients have bone pathology (5), indicating the potency of such regulators. CKD-MBD serves to highlight the mechanisms of bone mineralization in a context of disturbed phosphate homeostasis. FGF-23 and sclerostin production are elevated in osteocytes, and PTH is also elevated (6). The increase in FGF-23 is the result of changes in the ratio of inorganic phosphate to pyrophosphate in CKD (7). Chronic elevated PTH can promote osteoclast formation and demineralization of bone through the upregulation of RANK ligand (RANKL) and the downregulation of OPG through osteoblasts (increasing the RANKL:OPG), while sclerostin is a negative regulator of the anabolic Wnt signaling pathway (8).

Many diseases stemming from genetic mutations have contributed to the role of phosphate in bone mineralization, though the majority are mediated through a change in FGF-23 concentration or function. As previously discussed, autosomal dominant hypophosphatemic rickets is characterized by a mutation in FGF-23 that reduces its cleavage and increases circulating concentration, leading to phosphate wasting (9) from the reduction in renal

expression of NaPi-2a and NaPi-2c. A similar genetic disorder is X-linked hypophosphatemic rickets, which stems from a mutation in PHEX, which is involved in the regulation of normal concentrations of FGF-23. A mouse model of this disease has demonstrated that within PHEX activity, osteopontin fragments accumulate in bone and inhibit mineralization to produce osteomalacic bone (10). Another example is autosomal recessive hypophosphatemic rickets, which is the result of a mutation in DMP1. DMP1 controls osteocyte proliferation and facilitates mineralization by hydroxyapatite after being cleaved. Thus, impaired mineralization and osteocyte differentiation occur (9).

References

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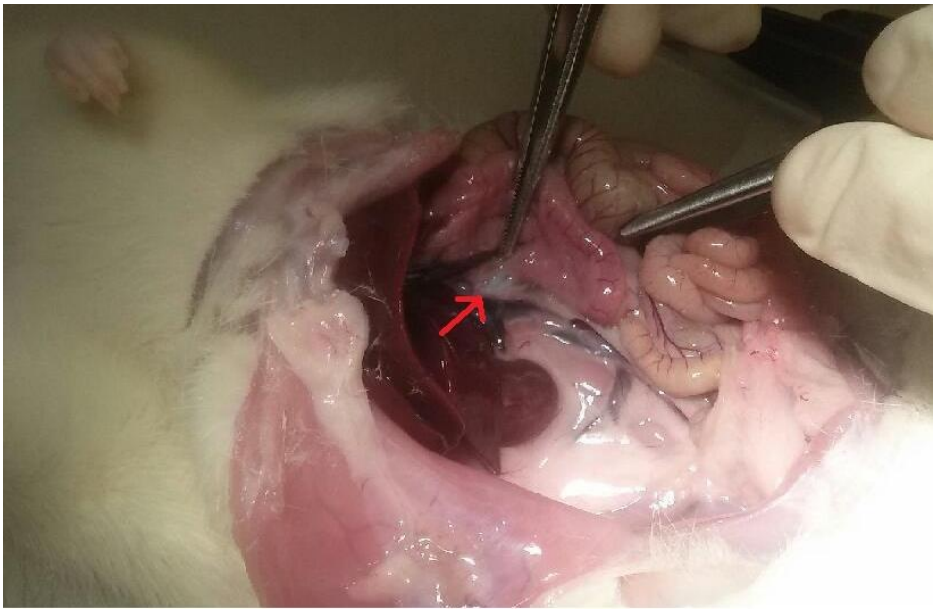
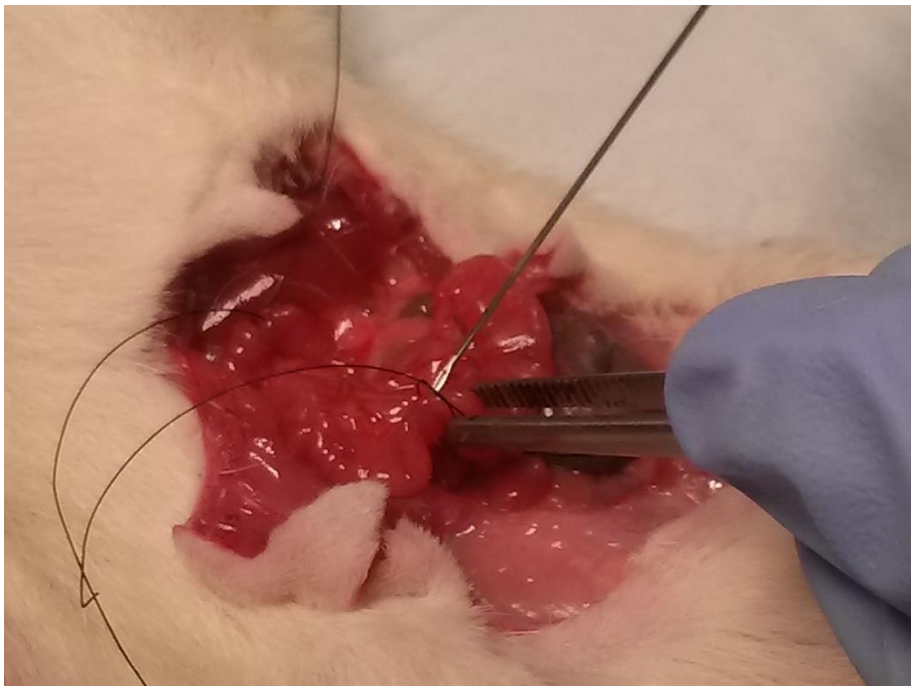
APPENDIX C: ADDITIONAL DETAILS ON THE LIGATED LOOP ABSORPTION METHOD

Protocol details with example photos:

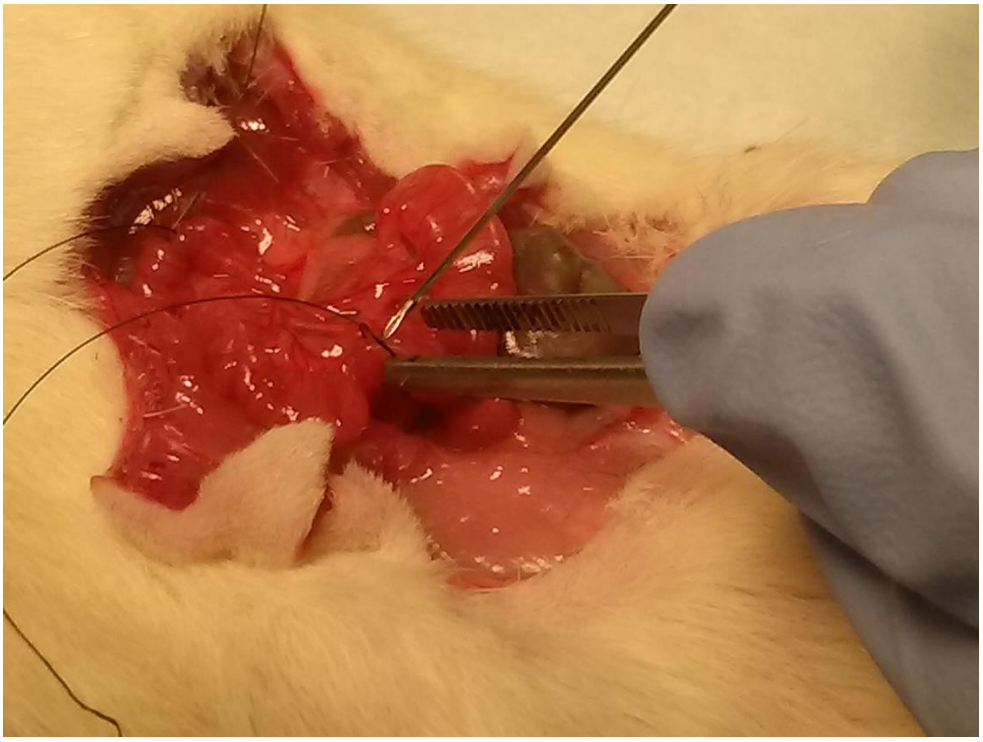
1) Prepare absorption buffers:
    a. Buffers with and without sodium are prepared according to concentrations from (1, 2). With sodium: HEPES sodium salt (16 mM), NaCl (140 mM), KCl (3.5 mM), KH_2PO_4 (0.1 mM). Without: HEPES free acid (16 mM), ChCl (140 mM), KCl (3.5 mM), KH_2PO_4 (0.1 mM).
    b. First, prepare buffers to 10X required concentration (ie 160 mM HEPES, 1400 mM NaCl or ChCl, 35 mM KCl, and 1 mM KH_2PO_4 in a 100 ml volumetric flask.
    c. Store this buffer solution at 4C as stock.
    d. To prepare doses, dilute stock solution 10X with ultrapure and P33 solution (based on number of injection doses required and accounting for P33 half life and days from certification). Mix well.
    e. Prepare P33 standard in duplicate by drawing 0.5ml of absorption buffer solution into Hamilton syringe and transferring into a scintillation vial with scintillation cocktail. Measure prior to experiment to verify expected radioactivity, and measure standards along with samples (collected as described below).
2) Draw 0.5ml of P33 solution into Hamilton syringe
3) Change gloves after handling P33 source
4) Check warm saline supply
5) Clean blades and tweezers with ethanol before each new rat
6) Anesthetize rat with isoflurane gas and **note time**.
7) Place jugular catheter and **note time**.
8) Ensure rat is under a heat lamp and/or on a heating pad
9) Connect syringe to catheter line, draw blood to syringe start (use this syringe for subsequent draws to syringe start during time draws)
10) Take baseline blood draw (0.4 or 0.5 ml – just be consistent between rats) and **note time**.
11) Connect new syringe to catheter line, push saline to clear line (only use this syringe to push new saline in)
12) Open rat, find caudal turn approximately 1cm distal to Ligament of Treitz (~8 cm distal to pyloric sphincter, the white suspensory tissue pictured below).

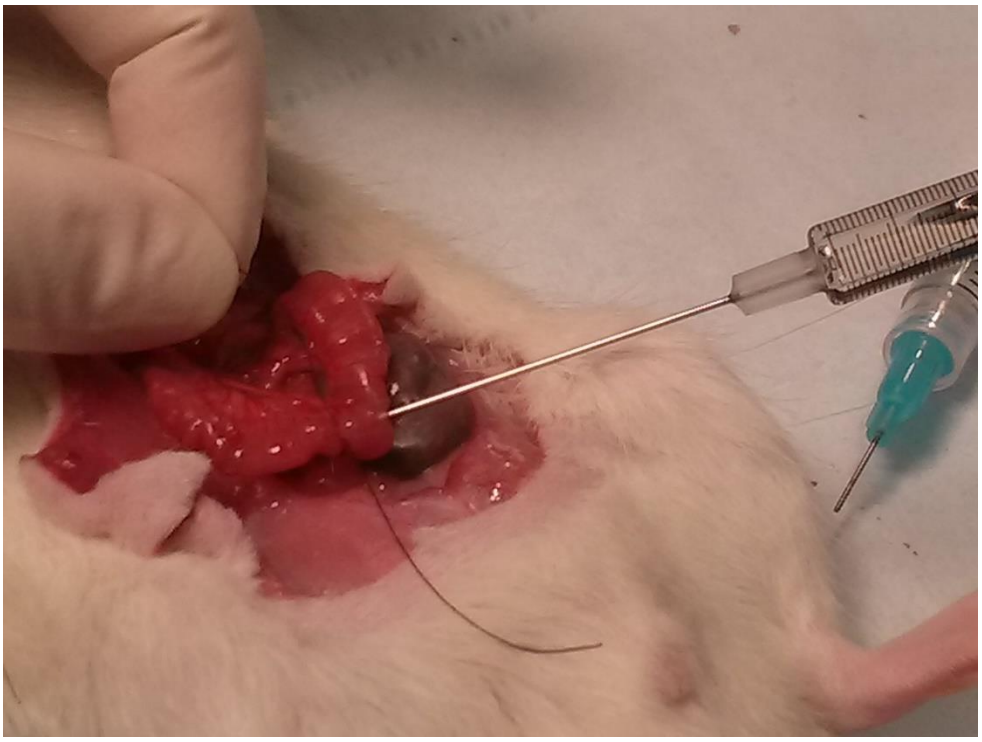


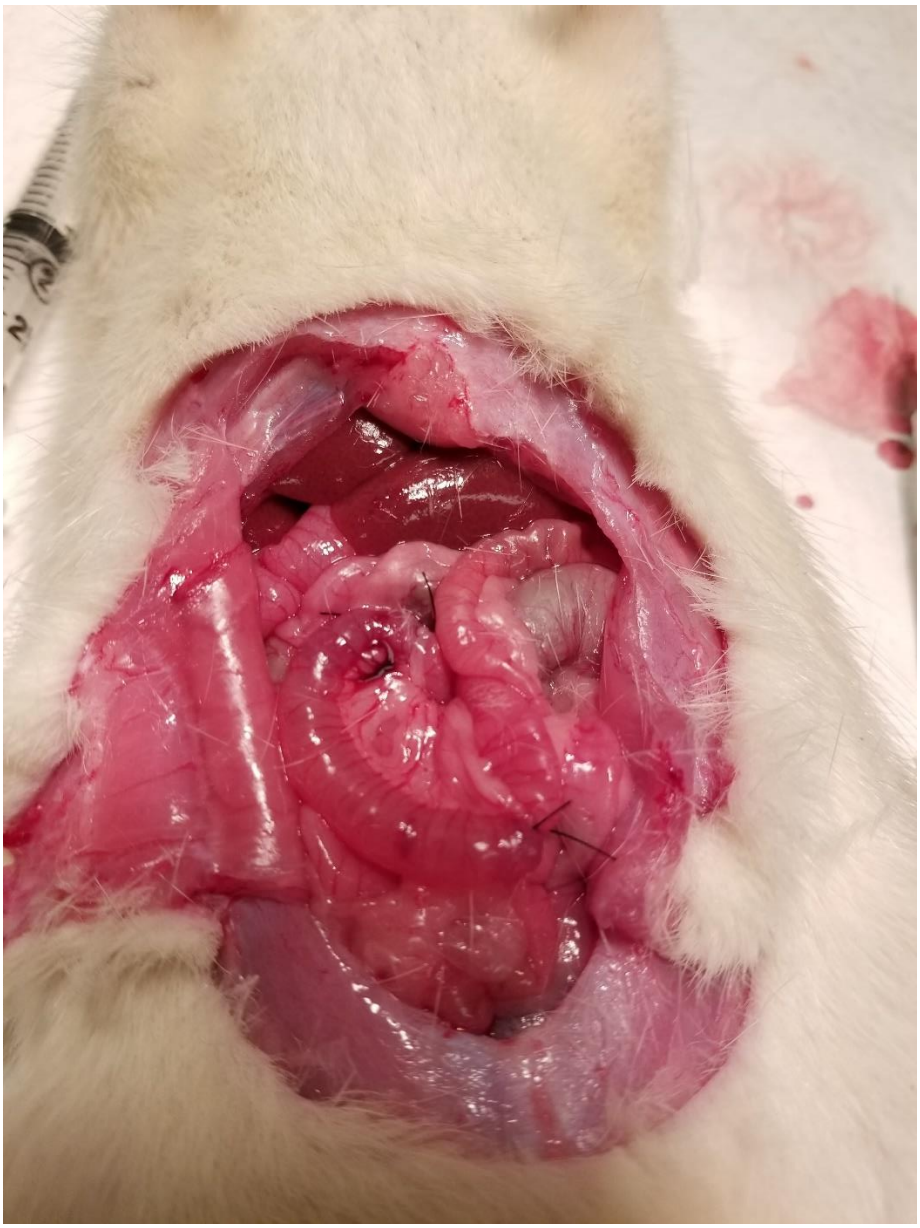
13) Place ligature, tighten enough to close tissue but not too tight so that it tears (2 knots)
14) Approximate 5cm distal to first knot, loosely place 2nd ligature. *Use a pre-measured 5cm piece of thread to use to measure the segment for consistency*



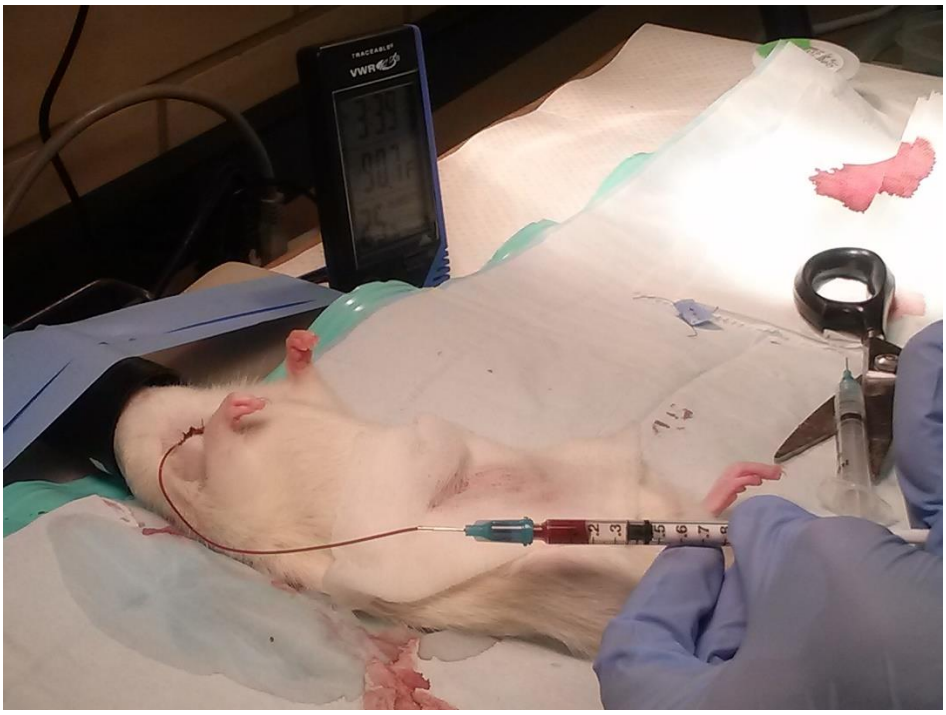
15) (2 people) With one person holding the 2 ends of the thread, inject Hamilton syringe through 2nd ligature, tighten loop over needle, inject slowly into loop, tighten ligature as much as possible over needle, and remove syringe and tighten quickly. Knot a second time and **note time**. Cut extra thread to ~1cm of the segment (otherwise when you solubilize the loop, the thread darkens the solution).







16) Change gloves to avoid radioactive contamination
17) Close rat and cover with warm, saline soaked gauze
18) **Write down** scheduled blood draw times on record sheet based on injection time
19) At 5, 10, 15, and 30 minutes after injection, repeat steps 8) – 10) to draw blood and **note times**.



20) After 30 minute draw, remove loop (maintain segment integrity) and **note length**, and place into 6 ml soluene-350 and **note time**.
21) Remove approximately 5cm of jejunum distal and 5 cm of duodenum proximal to where loop was for intestinal scraping.
22) Remove kidney and flash freeze in liquid nitrogen in foil
23) Take final blood draw- aim for 6ml and **note amount and time**.

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APPENDIX D: ADDITIONAL WORK

Diet and Fecal Digestion Methods Comparison for Phosphorus Recovery

Purpose of the project

Compare the recovery of phosphorus after various digestion methods.

1) Mixed diet spiked with disodium phosphate or sodium phytate
2) Wheat flour SRM (NIST)
3) Fecal sample spiked with ICP standard

Methods

Sample Descriptions

Mixed Diet

A mixed diet meal composite was made by blending the following foods: pork tenderloin, baked potato, sour cream, butter, baby carrots, baguette, applesauce, and skim milk. This diet was designed using the Nutrition Data System for Research 2007 (NDSR, Nutrition Coordinating Center [NCC], University of Minnesota, Minneapolis, MN, USA). During blending, it was spiked with either 1) no added P, 2) disodium phosphate, or 3) sodium phytate. For both the disodium phosphate and sodium phytate spikes, three different levels were used: low (0.45 mg P/g diet), medium (0.90 mg P/g diet), or high (1.35 mg P/g diet).

Wheat flour standard reference material (SRM)

Wheat flour SRM was purchased from NIST (SRM 1567b). Average P is certified by taking the average of the following analytical methods: DCP-AES, ICP-OES, SPECTRO, WDXRF.

Feces

A fecal homogenate was created by vortexing and inverting pooled stored human fecal homogenates from a previous experiment. The pooled fecal homogenate was spiked with

ammonium dihydrogen phosphate (ICP standard; SPEX CertiPrep) at 200 µL (200 ug P) or 400 µL (400 ug P) after samples were aliquoted for digestion.

Digestion Methods

Muffle Furnace Methods (MF600 and *MF500)*: diet or fecal samples were aliquoted into 40 mL crucibles (CoorsTek), weighed, and placed in a muffle furnace with lids (Thermolyne, Thermo Scientific). Temperature increased at 5 C/minute and held for 3 days at 600 C (*MF600*), or 2.5 days at 500 C (*MF500*). Crucibles were then removed from the furnace, allowed to cool overnight, weighed the next morning, and diluted 60X with 2% HNO_3 for analysis by inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT, USA). Lid were rinsed into the crucible during dilution to capture all phosphorus.

Microwave (MICRO): diet or fecal samples were weighed and aliquoted into vessels (MARS 6, CEM, Matthews, NC) 5 mL 70% HNO_3 and 5 ml water were added to each vessel for samples to begin digestion for 30 minutes. Vessels were then capped and placed in the microwave (MARS 6, CEM, Matthews, NC). The "One Touch" "food" method and a custom "feces" method were used with the following settings, and diluted 170X to 2% HNO_3:

"Food": ramp time 20:00-25:00 minutes, hold time 15:00 minutes, temperature 210 C.

"Feces": ramp time 20:00-25:00 minutes, hold time 15:00 minutes, temperature 200 C.

Short furnace and acid combination (COMB): samples were aliquoted into 40 mL crucibles (CoorsTek), weighed, and placed into the muffle furnace with lids. Temperature was ramped to 550 C and held for 1 hour. After cooling (~2 hours), 5 ml 70% HNO_3 was added to samples and heated in a water bath at 70 C for 15 minutes. Samples were then transferred to funnels with #5 filter paper (2.5 µm pore size) for filtration, and diluted with 2% HNO_3 for analysis on ICP-OES.

Perchloric acid digestion AOAC method (AOAC): diet or fecal samples were aliquoted into 50 mL graduated digestion glass tubes, and 5 mL 70% HNO_3 was added and covered with parafilm and samples were left overnight to digest. Under a percholoric-acid approved hood, 3 mL $HClO_4$ was added to each tube, and samples were heated in a heating block to ~175 C for > 50 ~ 90 minutes, then cooled. Samples were heated to ~200 C for 1.5 ~ 3 hours, until white fumes

disappeared. When tubes were cool, the solution was transferred to 50 mL Falcon tubes (Fisher) and diluted 600X with ultrapure water. Analysis was done using a colorimetric method on a spectrophotometer (PowerWaveX, Bio-Tek Instruments, Inc).

For each method, samples were prepared in triplicate or quadruplicate. Approximately 0.5 g diet and 1.5 g feces were used for all methods.

METHOD ABBREVIATION	DESCRIPTION	ACID USE FOR DIGESTION	MAX TEMPERATURE (°C)	TOTAL DIGESTION TIME	ANALYTICAL METHOD
MF600	Dry ash (muffle furnace)	-	600	3 days	ICP-OES
MF500	Dry ash (muffle furnace)	-	500	2.5 days	ICP-OES
MICRO	Microwave (CEM Mars 6)	35% HNO_3	210 (diet) 200 (feces)	1 h 20 minutes	ICP-OES
COMB	Dry ash (muffle furnace) + wet digestion (acid)	70% HNO_3	550	1 hour (furnace) Overnight (acid)	ICP-OES
AOAC	Wet digestion (HClO4 + HNO3), AOAC standard	70% HNO_3 + 70% $HClO_4$	200	~4.5 hours	Colorimetric

Table A.D.1. Parameters of each method tested for mixed diet, wheat flour, and fecal samples.

Statistics

Bland-Altman plots and 95% limits of agreement were generated with R 3.3.2 with the BlandAltmanLeh package for comparisons between methods. The plots reflect the mean difference between each method and the AOAC method, along with the estimation of the interval that 95% of the differences between the methods fall.

Results

Mixed diet:

- Methods 1, 2, 3, and 5 were similar in their measurement of baseline phosphorus. Method 4 was too low and too high.
- Recovery was closest to 100% with methods 1, 2, 3, and 5. Method 4 was low.

Baseline

Method 1	Method 2	Method 3	Method 4	Method 5
108 (9)	97 (13)	102 (19), 100 (23)	41 (21), 136 (15)	110 (8)

mg P/100g diet (+/- SD)

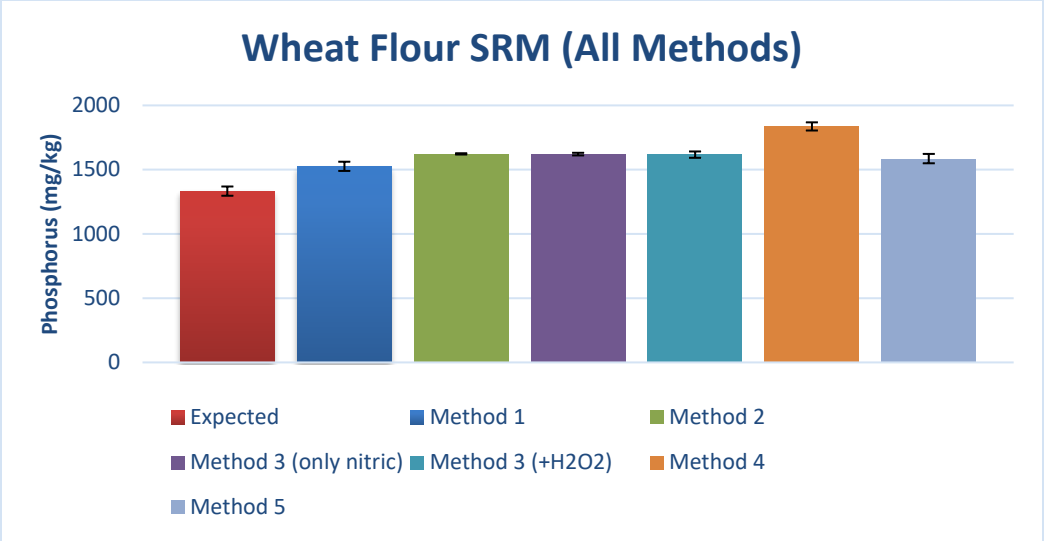
Recovery (% vs expected certified reference value)

Method	Disodium phosphate	Sodium phytate
1	104% (4%)	108% (2%)
2	99% (7%)	105% (6%)
3	95% (10%)	104% (7%)
3 (repeat 1)	113% (2%)	119% (2%)
3 (repeat 2)	104% (7%)	114% (3%)
4	47% (4%)	56% (5%)
4 (repeat)	75% (2%)	93% (2%)
5	103% (7%)	107% (7%)

(+/- SD)

Wheat flour:

- All methods were higher than expected (vs SRM certified value), but all methods except method 4 were similar to one another.



Note: method 3 was done twice, once with nitric acid only and one with nitric acid + hydrogen peroxide.

Recovery (% vs expected certified reference value)

Method	
1	114% (5%)
2	122% (0.7%)
3 (nitric acid only)	122% (2%)
3 (nitric + hydrogen peroxide)	121% (4%)
4	138% (5%)
5	119% (5%)

Fecal:

- Baseline: similar between method 1, 2, and 5. Method 3 slightly higher, method 4 highest.
- Recovery: Method 1 and 2 were a bit higher, methods 3, 4, and 5 were about the same, close to 100% recovery.

Baseline Fecal Phosphorus (before spike)

Method 1	Method 2	Method 3	Method 4	Method 5
2435 (340)	2431 (186)	2678 (126)	2950 (123)	2428 (97)

mg P/kg feces (+/- SD)

Recovery (% vs expected certified reference value)

Method	Low Spike	High Spike
1	110% (7%)	118% (5%)
2	111% (2%)	110% (5%)
3	102% (4%)	104% (2%)
4	97% (2%)	102% (6%)
5	96% (1%)	97% (7%)

(+/- SD)

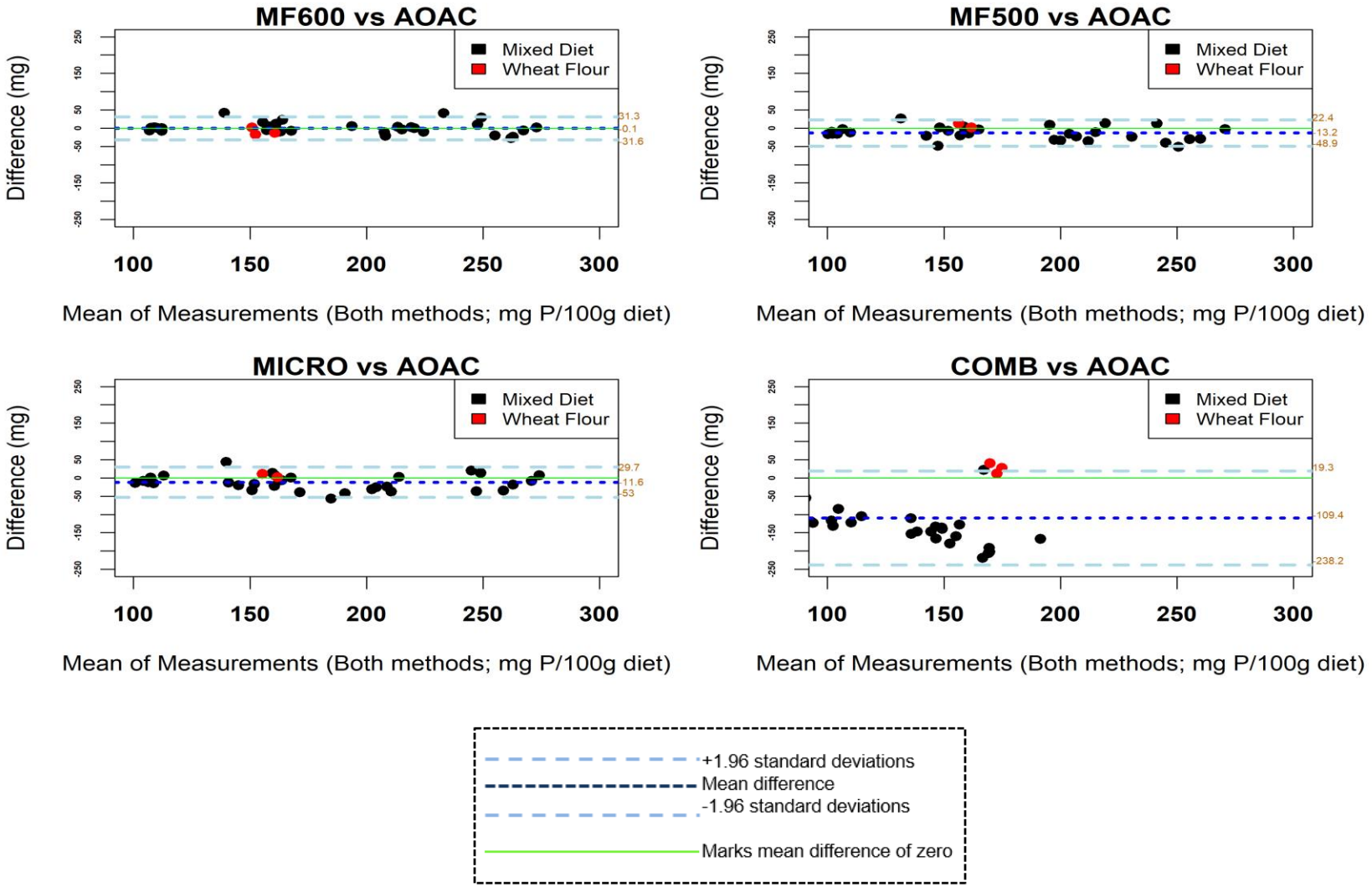


Figure A.D.1. Bland-Altman plots of each digestion method vs AOAC method for diet samples. MF600 had the highest consistency with AOAC with the tightest limits of agreement (mean difference -0.1 mg). The MICRO and MF500 methods had similar consistency

Figure 1. continued

(mean difference -13.2 and -11.6 mg). COMB had poor recovery of phosphorus vs AOAC and is not a suitable method for digestion of mixed diet samples. Phosphorus in wheat flour was similar to AOAC with MF600, MF500, and MICRO.

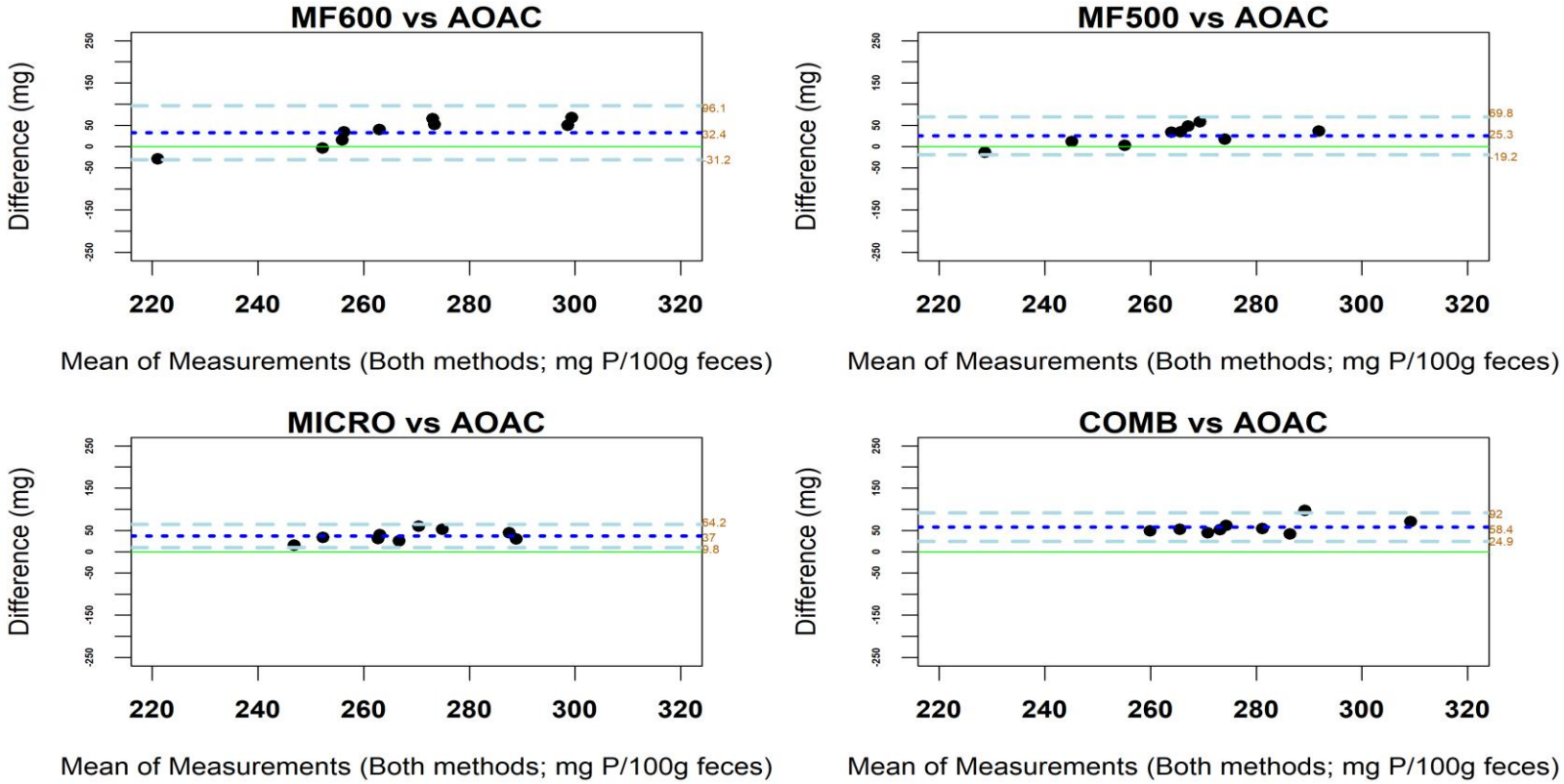


Figure A.D.2. Bland-Altman plots of each digestion method vs AOAC method for human fecal samples. Mean differences for MF600 and MF500 compared to AOAC were similar at 32.4 and 25.3 mg phosphorus. The 95% limits of agreement did not include zero for MICRO or COMB, though mean differences were similar to other methods

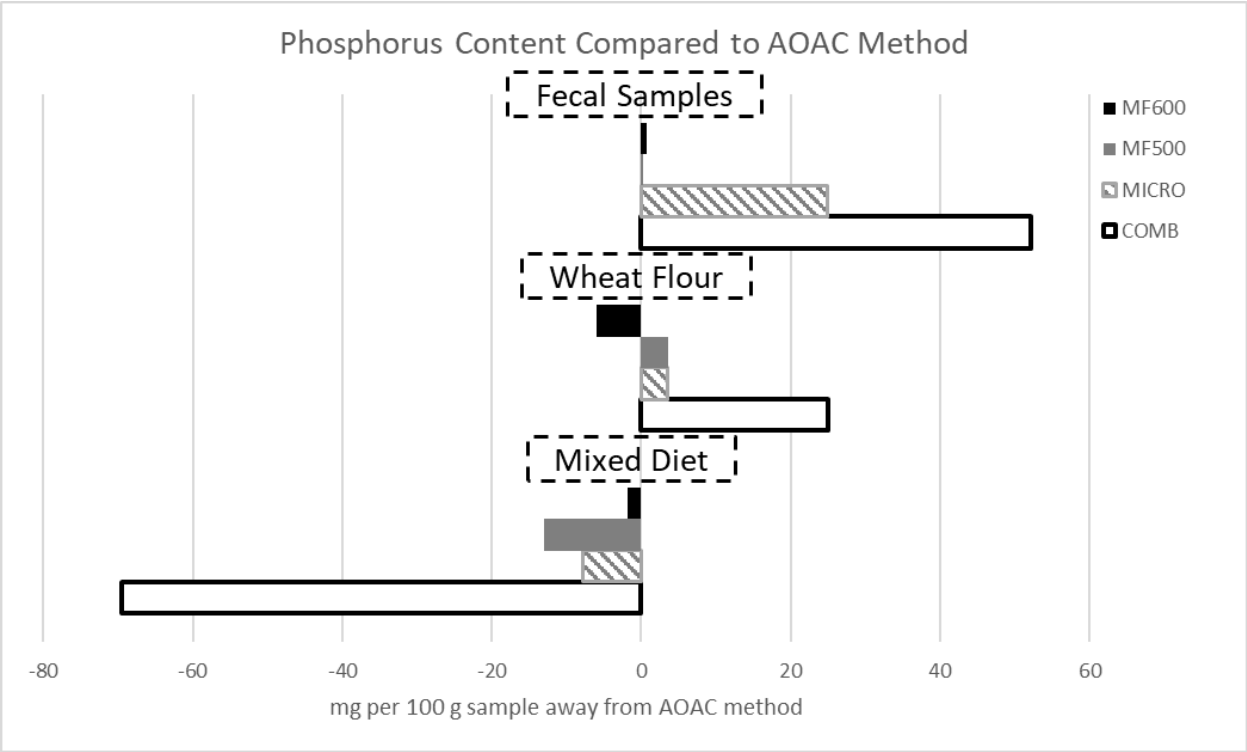


Figure A.D.3. Overall average differences for each digestion method compared to the AOAC method for mixed diet, wheat flour, and fecal samples.

Conclusions

Diet Digestion

- MF600, MICRO, and MF500 are suitable alternatives to the AOAC nitric acid+perchloric acid digestion for measurement of phosphorus in mixed diet samples and wheat flour. Recovery of spiked phosphorus confirmed these results.
- COMB (short dry digestion + nitric acid digestion) is not suitable for mixed diet sample analysis.

Human Fecal Digestion

- MF600 and MF500 are most consistent with the AOAC method for phosphorous measurement in fecal samples. MICRO and COMB slightly overestimated phosphorus but are suitable alternatives. Recovery of spike largely corroborated these results.

Overall conclusions

Muffle furnace at 600 or 500 C, or microwave digestion of diet and human feces are reliable alternatives to the AOAC method. Such methods provide additional choices to the use of the hazardous chemical perchloric acid required by the AOAC method. Additional support for the use of microwave digestion of foods and measurement of phosphorus by ICP-OES has been published and adopted as a new AOAC method (1).

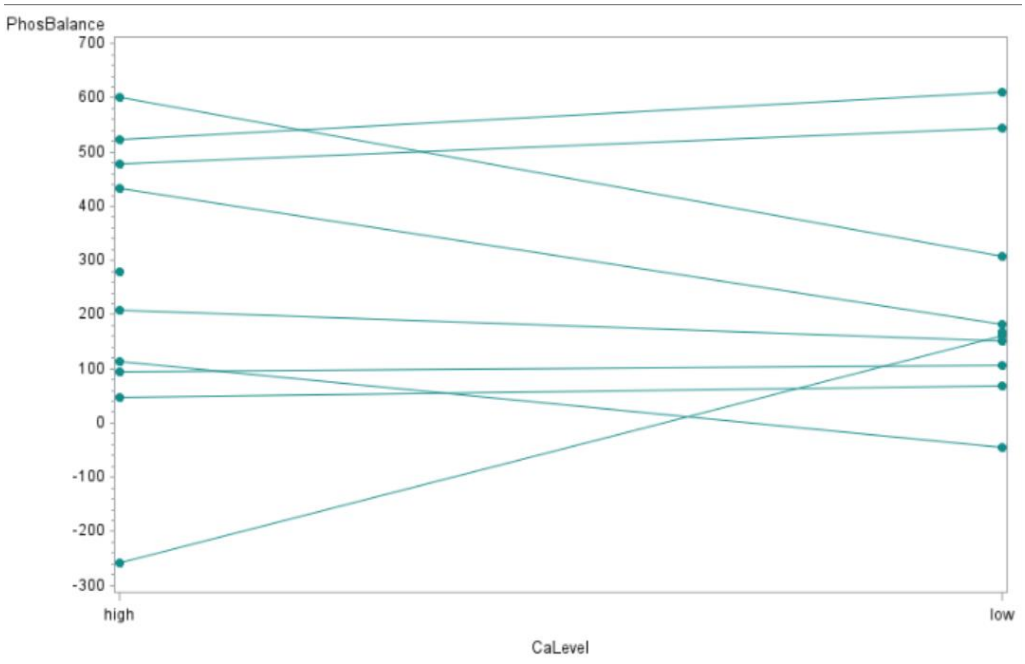
Reference

1. Poitevin E. Determination of calcium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium, and zinc in fortified food products by microwave digestion and inductively coupled plasma-optical emission spectrometry: Single-laboratory validation and ring trial. Journal of AOAC International 2012;95(1):177-85.

APPENDIX E: EXAMPLE SAS CODE FROM PRIMARY OUTCOMES

Appendix E1: Phosphorus Balance and Net Phosphorus Absorption in Adolescent Females

```
symbol1 color=vibg interpol=join value=dot;
symbol2 color=vibg interpol=join value=dot;
symbol3 color=vibg interpol=join value=dot;
symbol4 color=vibg interpol=join value=dot;
symbol5 color=vibg interpol=join value=dot;
symbol6 color=vibg interpol=join value=dot;
symbol7 color=vibg interpol=join value=dot;
symbol8 color=vibg interpol=join value=dot;
symbol9 color=vibg interpol=join value=dot;
symbol10 color=vibg interpol=join value=dot;
symbol11 color=vibg interpol=join value=dot;
proc gplot data=a1;
plot PhosBalance*CaLevel = id /regeqn;
run;
```




```
proc mixed;
class order id period CaLevel;
model NetPhosAbs = order CaLevel Period;
random id;
lsmeans CaLevel;
run;
```

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
order	1	7	0.11	0.7499
CaLevel	1	7	1.72	0.2305
period	1	7	0.00	0.9699

Least Squares Means

Effect	CaLevel	Estimate	Standard Error	DF	t Value	Pr > |t|
CaLevel	**high**	773.96	64.3389	7	12.03	<.0001
CaLevel	**low**	875.75	62.0103	7	14.12	<.0001

```
proc mixed;
class order id period CaLevel;
model PercNetPhosAbs = order CaLevel Period;
random id;
lsmeans CaLevel; run;
```

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
order	1	7	0.05	0.8346
CaLevel	1	7	2.16	0.1855
period	1	7	0.02	0.9000

Least Squares Means

Effect	CaLevel	Estimate	Standard Error	DF	t Value	Pr > |t|
CaLevel	**high**	0.5332	0.04313	7	12.36	<.0001
CaLevel	**low**	0.6119	0.04153	7	14.73	<.0001

Appendix E2: Phosphorus Absorption Efficiency, Disappearance from Ligated Loop, in Healthy Male Sprague Dawley Rats

```
proc glm data=loops;
class age level;
model y = age level age*level;
```

```
lsmeans age level age*level / pdiff adjust=tukey;
run;
```

The GLM Procedure

Dependent Variable: y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.11355494	0.01419437	2.66	0.0141
Error	61	0.32497131	0.00532740		
Corrected Total	69	0.43852625			

R-Square	Coeff Var	Root MSE	y Mean
0.258947	19.41544	0.072989	0.375933

Source	DF	Type I SS	Mean Square	F Value	Pr > F
age	2	0.08439165	0.04219583	7.92	0.0009
level	2	0.00739157	0.00369578	0.69	0.5036
age*level	4	0.02177172	0.00544293	1.02	0.4034

Source	DF	Type III SS	Mean Square	F Value	Pr > F
age	2	0.08087616	0.04043808	7.59	0.0011
level	2	0.00767372	0.00383686	0.72	0.4907
age*level	4	0.02177172	0.00544293	1.02	0.4034

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

age	y LSMEAN	LSMEAN Number
10	0.42397024	1
20	0.35588750	2
30	0.34760774	3

Least Squares Means for effect age
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: y

i/j	1	2	3
1		0.0062	0.0022
2	0.0062		0.9203
3	0.0022	0.9203	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

level	y LSMEAN	LSMEAN Number
High	0.36674524	1
Low	0.39039583	2
Normal	0.37032440	3

Least Squares Means for effect level
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: y

i/j	1	2	3
1		0.5120	0.9849
2	0.5120		0.6164
3	0.9849	0.6164	

Appendix E3: Phosphorus Absorption Efficiency, Disappearance from Ligated Loop, in Cy/+ Male Rats

```
proc glm data=loops_dependent;
class genotype buffer age;
model abs = genotype age age*genotype;
lsmeans age genotype age*genotype / pdiff adjust=tukey STDERR;
run;
```

The GLM Procedure

Dependent Variable: abs

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.09199653	0.03066551	10.89	<.0001
Error	44	0.12387478	0.00281534		
Corrected Total	47	0.21587132			

R-Square	Coeff Var	Root MSE	abs Mean
0.426164	16.73612	0.053060	0.317037

Source	DF	Type I SS	Mean Square	F Value	Pr > F
genotype	1	0.01448764	0.01448764	5.15	0.0283
age	1	0.07705914	0.07705914	27.37	<.0001
genotype*age	1	0.00044975	0.00044975	0.16	0.6913

Source	DF	Type III SS	Mean Square	F Value	Pr > F
genotype	1	0.01448764	0.01448764	5.15	0.0283
age	1	0.07705914	0.07705914	27.37	<.0001
genotype*age	1	0.00044975	0.00044975	0.16	0.6913

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

genotype	age	abs LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
CY	**20**	0.37753884	0.01531703	<.0001	1
CY	**30**	0.29128194	0.01531703	<.0001	2
Norm	**20**	0.33667053	0.01531703	<.0001	3
Norm	**30**	0.26265772	0.01531703	<.0001	4

Least Squares Means for effect genotype*age
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: abs

i/j	1	2	3	4
1		0.0014	0.2485	<.0001
2	0.0014		0.1705	0.5545
3	0.2485	0.1705		0.0072
4	<.0001	0.5545	0.0072	

Appendix E4: Blood Urea Nitrogen Over Time from Cy/+ Female Rats that Underwent Ovariectomy or Sham Surgery

```
proc glm data=BUN;
class OVX;
model Time0--Time4 =
      OVX / nouni;
repeated Time 5 (0 1 2 3 4) polynomial / summary printe;
run;
```

The GLM Procedure
Repeated Measures Analysis of Variance
Univariate Tests of Hypotheses for Within Subject Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F	Adj Pr > F	
						G - G	H-F-L
Time	4	47.1384675	11.7846169	3.83	0.0081	0.0162	0.0084
Time*OVX	4	20.3021825	5.0755456	1.65	0.1752	0.1924	0.1760
Error(Time)	56	172.5167100	3.0806555				

Greenhouse-Geisser Epsilon	0.7565
Huynh-Feldt-Lecoutre Epsilon	0.9885

Appendix E5: Bland-Altman Plot Comparing Phosphorus Digestion Methods (done in R)

```
library(ggplot2)
library(BlandAltmanLeh)

df<-read.csv("T:/../Hill Gallant lab/Methods Comp/data for R.csv",sep = ",")

material <- df$Material

ba.stats <- bland.altman.stats(df$MF600.all, df$AOAC.all)

plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF600 vs AOAC", ylim=c(-250,250), pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.7, font =2)
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2)


#########diet######

png(filename="T:/../Hill Gallant Lab/Colby/Diet Phos Methods
Comparison/R/Std_PNG.png",
    units="in",
    width=12,
    height=7,
    pointsize=10,
    res=300)
par(mfrow=c(2,2),cex=1.5,mar=c(5, 4, 1, 2.5) + 0.1)#it goes c(bottom, left,
top, right) )
material <- df$Material

ba.stats <- bland.altman.stats(df$MF600.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF600 vs AOAC", xlim=c(100,300), ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
#print(ba.stats$lines)
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=35
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
```

```
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=c(0)
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-35
)
abline(h=0, col="green")
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2,
cex=0.8)

ba.stats <- bland.altman.stats(df$MF500.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF500 vs AOAC", xlim=c(100,300),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=30
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=c(-10)
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-40
)
abline(h=0, col="green")
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2,
cex=0.8)

ba.stats <- bland.altman.stats(df$MICRO.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
```

```
     main="MICRO vs AOAC", xlim=c(100,300),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=30
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=c(-10)
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-40
)
abline(h=0, col="green")
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2,
cex=0.8)

ba.stats <- bland.altman.stats(df$COMB.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="COMB vs AOAC", xlim=c(100,300),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=30
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-100
)
```

```
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-225
)
abline(h=0, col="green")
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2,
cex=0.8)

dev.off()

################

df<-read.csv("T:/../Hill Gallant lab/Methods Comp/data for R.csv",sep = ",")

material <- df$Material

ba.stats <- bland.altman.stats(df$MF600.all, df$AOAC.all)

plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g diet)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF600 vs AOAC", ylim=c(-250,250), pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.7, font =2)
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
legend(x = "topright", legend = c("Mixed Diet","Wheat Flour"), fill = 1:2)


#########feces######

df<-read.csv("T:/../Hill Gallant lab/Methods Comp/data for R - feces.csv",sep
= ",")


png(filename="T:/../Hill Gallant Lab/Colby/Diet Phos Methods
Comparison/R/rplot feces.png",
    units="in",
    width=12,
    height=7,
    pointsize=10,
    res=300)
par(mfrow=c(2,2),cex=1.5,mar=c(5, 4, 1, 2.5) + 0.1)#it goes c(bottom, left,
top, right) )

ba.stats <- bland.altman.stats(df$MF600.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g feces)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF600 vs AOAC", xlim=c(220,320), ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
```

```
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
#print(ba.stats$lines)
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=92
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=30
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=-30
)
abline(h=0, col="green")

ba.stats <- bland.altman.stats(df$MF500.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g feces)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MF500 vs AOAC", xlim=c(220,320),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=80
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=30
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
```

```
      at=-23
)
abline(h=0, col="green")

ba.stats <- bland.altman.stats(df$MICRO.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g feces)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="MICRO vs AOAC", xlim=c(220,320),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=70
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=35
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=7
)
abline(h=0, col="green")

ba.stats <- bland.altman.stats(df$COMB.all, df$AOAC.all)
plot(ba.stats$means, ba.stats$diffs, xlab="Mean of Measurements (Both
methods; mg P/100g feces)", ylab="Difference
(mg)",cex.lab=1,col.lab="Black",col=material,
     main="COMB vs AOAC", xlim=c(220,320),ylim=c(-250,250),
pch=19,xaxt="n",yaxt="n")
axis(1, cex.axis = 1, font = 2)
axis(2, cex.axis = 0.4, font =2, at=c(seq(from=-250,to=250,by=50)))
abline(h = ba.stats$lines, lty=c(2,3,2),
col=c("lightblue","blue","lightblue"),
       lwd=c(3,3,3))
mtext(text=round(ba.stats$lines['upper.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=90
)
mtext(text=round(ba.stats$lines['mean.diffs'],digits=1), las=1,
```

```
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=54
)
mtext(text=round(ba.stats$lines['lower.limit'],digits=1), las=1,
      side=4,
      outer = FALSE,
      cex = 0.7,
      col = "#B36000",
      at=25
)
abline(h=0, col="green")
#L3uq2kyrfqrm3VC5rFEzqHiEiZqAACjZRm4fjdBprafCxVmrQcEz#
dev.off()
```

APPENDIX F: EXTERNAL ABSTRACTS AND POSTERS FROM DISSERTATION PROJECT

Appendix F1: ASBMR Annual Meeting 2014, Houston, TX

Effect of dietary calcium on phosphorus balance and net absorption in healthy adolescent girls

Phosphorus is an essential nutrient which plays a critical role in energy metabolism as ATP and as a major structural component of bone. Phosphorus deficiencies are extremely rare as it is widespread in the food supply. Conversely, there is increasing concern over potential harms of dietary phosphorus excess. Inorganic forms of phosphorus are commonly used as food additives, which contributes to an increased intake in the U.S. Emerging evidence shows that elevated serum phosphorus and high dietary phosphorus intake may increase the risk of cardiovascular disease and mortality in patients with chronic kidney disease as well as the general population. However, few phosphorus balance studies have been conducted, and are needed to examine factors that influence whole body phosphorus metabolism.

The purpose of this study was to determine the effect of dietary calcium on phosphorus balance and net phosphorus absorption in healthy adolescent girls, utilizing a unique resource of banked urine, fecal, and diet samples from a controlled calcium balance study previously conducted at Purdue University. Eleven healthy girls ages 11-14y participated in a randomized crossover study conducted in 2007 which consisted of two 3-week periods of a controlled diet with low (817 ± 62 mg/d) or high (1418 ± 35 mg/d) calcium, separated by a 1-week washout period. Phosphorus intake was the same on the low and high calcium diets (1531 ± 29 and 1534 ± 30 mg/d, respectively, $p = 0.83$). Results show urinary phosphorus excretion was lower on the high calcium diet (649 ± 41 vs 535 ± 42 mg/d, $p = 0.01$). However, fecal phosphorus (553 ± 60 vs 678 ± 63 vs mg/d, $p = 0.14$), net phosphorus absorption (980 ± 56 vs 859 ± 58 mg/d, $p = 0.14$), and overall phosphorus balance (339 ± 72 vs 329 ± 74 mg/d, $p = 0.90$) were not significantly different between low and high calcium intake. This agrees with our previously published study of phosphorus balance in adult moderate-stage chronic kidney disease patients, where increased calcium intake modestly reduced urinary phosphorus, but did not affect overall

phosphorus balance or absorption. Combined, these balance studies suggest that increasing dietary calcium is not an effective strategy for reducing phosphorus absorption or retention.

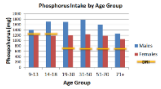
Effect of Dietary Calcium on Phosphorus Balance and Net Absorption in Healthy Adolescent Girls

Colby J. Vorland[1], Berdine R. Martin[1], Connie M. Weaver[1], Munro Peacock[2], Kathleen M. Hill Gallant[1].
[1]Department of Nutrition Science, Purdue University, W. Lafayette, IN. [2]Department of Medicine, Indiana University, Indianapolis, IN.

INTRODUCTION

- Phosphorus (P) is an essential nutrient that has critical roles in the body such as in energy metabolism as ATP and structural function in bones.
- P is widespread in the food supply, therefore deficiencies are extremely rare. Conversely, there is increasing concern over potential harms of dietary P excess.

Figure 1. Average P Intake by Age. The average dietary intake of P is well above the Recommended Dietary Allowance for adults over the age of 18. *(NHANES '09-'10, Day 1 Dietary Intake, Weighted)*



- Inorganic forms of P are commonly used as food additives, and this has contributed to an increased intake in the U.S. Nutrient databases may not accurately reflect this.

Figure 2. Elevated P Increases Cardiovascular and Bone Disease Risk. Emerging evidence shows that elevated serum P and dietary P intake may increase the risk of cardiovascular disease, bone disease, and mortality in patients with chronic kidney disease (CKD) as well as the general population. *(Moe SM et al. 2009 Kidney Int Suppl; 113:S1-130)*

- Calcium-based P binders are used in CKD to prevent P absorption and retention. However, few phosphorus balance studies have been conducted to determine the effects of calcium on whole body phosphorus retention.
- Our recent balance study in moderate-stage CKD patients found that calcium carbonate does not reduce P absorption or alter balance, but significantly increases calcium retention[2]. Additional data on the effects of calcium on whole body phosphorus retention is needed.

OBJECTIVE

The purpose of this study was to determine the effect of dietary calcium on phosphorus balance and net phosphorus absorption in healthy adolescent girls, utilizing a unique resource of banked samples from controlled calcium balance studies previously conducted at Purdue University. It was hypothesized that an increase in dietary calcium would reduce phosphorus absorption, urine phosphorus, and phosphorus balance.

METHODS

Banked fecal and urine samples from 11 adolescent girls who participated in controlled calcium balance studies conducted at Purdue University in 2007 were analyzed for phosphorus content.

Diet

Subjects consumed a controlled diet and were assigned to receive added calcium from calcium carbonate ("high calcium") or placebo ("low calcium") in a randomized cross-over design.

High Calcium diet: 1418 ± 35 mg per day **Low Calcium diet**: 817 ± 62 mg per day

Design

Each 3-week study session consisted of 1 week equilibration followed by a 2 week balance period when all urine and fecal samples were collected.

METHODS

The purpose of the original 2007 study was to investigate the effects of calcium carbonate particle size calcium absorption and balance[1]. Dietary P was estimated from controlled diet menus with NDSR software, and actual dietary P was determined by inductively coupled plasma spectrophotometry (ICP-OES).

Urine and fecal P and Ca were determined by ICP-OES. Urine P and Ca were corrected for average urine creatinine excretion.

Mineral Balance

Mineral balance was calculated as:
Intake P or Ca (mg/d) – urine P or Ca (mg/d) – fecal P or Ca (mg/d)

Fractional net mineral absorption was calculated as:
(Intake P or Ca (mg/d) – fecal P or Ca (mg/d))/Intake P or Ca (mg/d)

Repeated measures ANOVA for cross-over designs using the PROC MIXED procedure with subject as a random effect was conducted in SAS version 9.3.

RESULTS

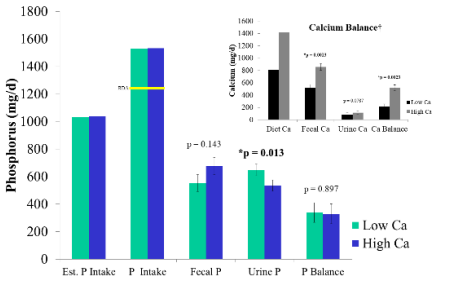


Figure 1. Calcium Intake on Phosphorus Balance. Measured P intake was 35% greater than that estimated by NDSR. Urinary P was lower on the high calcium diet, while fecal P and P balance were not different. †Calcium balance results, previously published[1] are presented above.

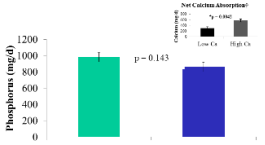


Figure 2. Net Phosphorus Absorption is Unchanged by Calcium Intake. †Calcium net absorption, previously published[1] is above.

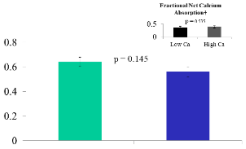


Figure 3. Fractional Net Phosphorus Absorption is Unchanged by Calcium Intake. †Calcium fractional net calcium absorption, previously published[2] is above.

RESULTS

Ethnicity (n)	Hispanic 1	Non Hispanic 10
Race (n)	White 10	Asian 1
Tanner stage (n for stages 1-5)	0/1/1/0/7	
Age (y)	13.5 ± 1.0	
Height for age (%ile)	48.9 ± 29.1	
Weight for age (%ile)	60.1 ± 32.8	
BMI (kg/m²)	21.7 ± 4.7	
BMI for age (%ile)	62.3 ± 34.2	

Mean ± SD unless otherwise noted.
Tanner stage data missing for two participants.

Table 1. Baseline Participant Characteristics

SUMMARY & CONCLUSIONS

- Urinary phosphorus is reduced on a high calcium diet in healthy young girls (1.9 mg phosphorus per 10 mg calcium; Figure 1).
- Fecal phosphorus, net absorption, and overall balance are not affected by increased calcium intake (Figures 1-3).
- The underestimation of dietary phosphorus by a nutrient database by 33% is in the range of other studies[3] (Figure 1).
- These results agree with a previous study in adult moderate-stage chronic kidney disease patients, where increased calcium intake modestly reduced urinary phosphorus, but did not affect overall phosphorus balance or absorption[2].

These balance studies suggest that manipulating dietary calcium may not be an effective way to alter phosphorus absorption or retention.

References

1. Elhle, A. E., Hill, K. M., Park, C. Y., Martin, B. R., Peacock, M., & Weaver, C. M. (2011). Effect of calcium carbonate particle size on calcium absorption and retention in adolescent girls. *Journal of the American College of Nutrition,30*(3), 171-177.

2. Hill, K. M., Martin, B. R., Wastney, M. E., McCabe, G. P., Moe, S. M., Weaver, C. M., & Peacock, M. (2013). Oral calcium carbonate affects calcium but not phosphorus balance in stage 3–4 chronic kidney disease. *Kidney International*, 83(5), 959-966.

3. Calvo, M. S., Moshfegh, A. J., & Tucker, K. L. (2014). Assessing the health impact of phosphorus in the food supply: issues and considerations. *Advances in Nutrition*, 5(1), 104-113.

Appendix F2: Indiana Musculoskeletal Symposium 2016, Indianapolis, IN

Effect of Age and Dietary Phosphorus Intake on Intestinal Phosphorus Absorption in Male Rats

Hyperphosphatemia is associated with negative bone outcomes in Chronic Kidney Disease. Improving the understanding of the regulation of intestinal phosphorus absorption may improve treatment strategies targeting absorption. The purpose of this study was to determine the effects of age and dietary phosphorus intake level on intestinal phosphorus absorption efficiency in male rats. Seventy-two male Sprague Dawley rats, ages 8-weeks, 18-weeks, and ~28-weeks were randomized into defined diets with 0.6% calcium and three different levels of phosphorus: 0.1% (low), 0.6% (normal), or 1.2% (high) for 14 days (n = 8 rats per age and diet group). Rats were housed individually in metabolic cages, and feces, urine, and diet were collected, weighed, and analyzed over the final four days of the study to calculate phosphorus balance. On day 14, phosphorus absorption efficiency was assessed using ligated loops of jejunum. (5 microCi P^{33} in 0.5 mL of transport buffer (0.1 mmol/L phosphate). Serial blood samples were collected over 30 minutes, and the jejunal loop was excised at 30 minutes. Plasma samples and the digested jejunal loop were analyzed for P^{33} activity by scintillation counting. Two-way analysis of variance was used to determine main effects (age and diet) and the interaction effect for age x diet, with Tukey's post-hoc comparisons.

Results show that phosphorus appearance into the plasma was higher in the 10-week old rats compared to the 20- and 30-week rats at 30 minutes (0.073 vs 0.052 and 0.048% of initial dose, respectively, $p < 0.0001$ for both comparisons). Similarly, % absorption, determined from disappearance of P^{33} activity from the intestinal loop at 30 minutes, was higher in the 10-week old rats compared with 20- and 30-week old rats (40.1 vs 34.7 and 34.6% of total dose, respectively, $p = 0.01$ for both). There were no differences in absorption efficiency between the three levels of dietary phosphorus ($p = 0.65$), and no age x diet interaction. Ten-week old rats had a higher positive phosphorus balance vs 20- and 30-week (37.2 vs 6.5 and 9.8 mg/day, respectively, $p = 0.0008$ and $p = 0.0013$), while the high phosphorus intake group had a higher positive balance than the normal and low intakes (31.7 vs 10.4 and 11.2 mg/day, respectively, $p = 0.01$ and $p = 0.03$). Together, these results suggest that intestinal phosphorus absorption is more efficient at younger ages in rats, but that changes in dietary phosphorus do not elicit adaptation in the efficiency of jejunal phosphorus absorption.

Effect of age and dietary phosphorus intake on intestinal phosphorus absorption in male rats

Colby J. Vorland, Pamela Lachcik, James Fleet, Kathleen M. Hill Gallant.
Department of Nutrition Science, Purdue University, W. Lafayette, IN.

PURDUE UNIVERSITY

Introduction

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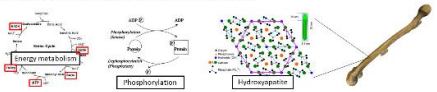
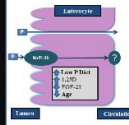


However, there is increasing concern that elevated serum and dietary P increase cardiovascular disease risk and mortality.



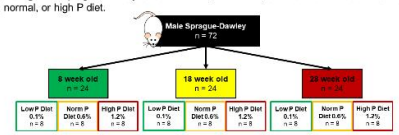
Intestinal P absorption is understood to occur by active (largely through the transporter NaPi-IIb) and paracellular pathways. In vitro evidence suggests that the level of dietary phosphorus and age affect NaPi-IIb expression and uptake into the enterocyte. However, absorption in response to these factors has not been tested in a more physiological context. (*Marks J et al. 2013 Curr Opin Nephrol Hypertens.; 22:481*)

Our goal is to improve the understanding of how active intestinal P absorption *adapts* to different factors. Therefore our primary aims were to:

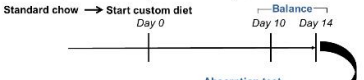
- **Examine the effect of altering the level of dietary P intake and age on in situ intestinal P absorption efficiency and whole-body P balance in healthy rats.**

Methods

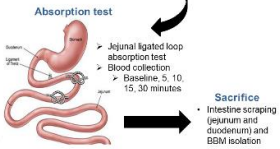
Study design: 72 Sprague-Dawley rats of 8 weeks, 18 weeks, and 28 weeks were randomly assigned a low, normal, or high P diet.



Diet: Rats were placed on the assigned diet for 14 days, while **balance** was done on days 10-14.



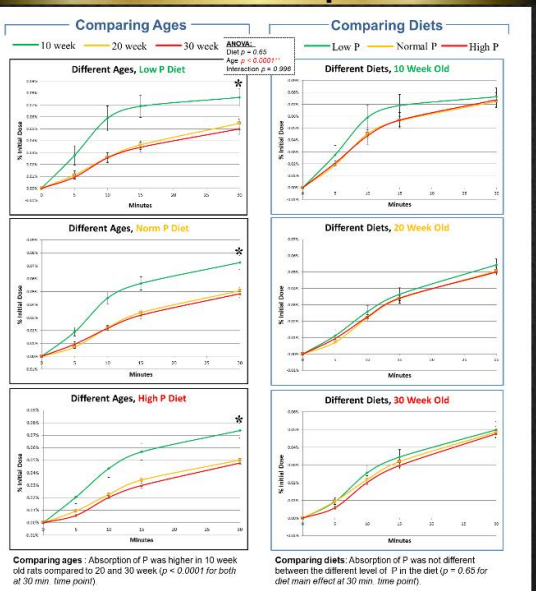
Absorption test: 5 µCi ^{33}P was injected into a 5cm jejunal loop, and appearance into the plasma as well as ^{33}P remaining in the loop at 30 minutes were measured to quantify absorption.



% Efficiency: Absorption efficiency was calculated as:
(1-(^{33}P remaining in loop)/Total ^{33}P dose)

Statistics: 2-way ANOVA was performed for all outcomes with the main effects and interaction of diet and age. Post-hoc comparisons were evaluated with the Tukey test.

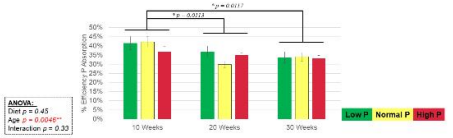
Results: Absorption



Comparing ages : Absorption of P was higher in 10 week old rats compared to 20 and 30 week ($p < 0.0001$ *for both at 30 min. time point*).

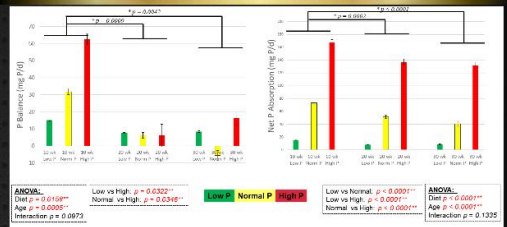
Comparing diets: Absorption of P was not different between the different level of P in the diet ($p = 0.65$ *for diet main effect at 30 min. time point*).

Absorption into plasma: Absorption assessed by appearance of ^{33}P in plasma, expressed as % of initial dose.



Absorption efficiency from loop: Efficiency was higher in 10 week old rats compared to 20 and 30 ($p = 0.0113$ *and* $p = 0.0117$), but there were no differences between level of P in the diet ($p = 0.45$ *for diet main effect*), confirming the plasma absorption results. Absorption efficiency (%) assessed by disappearance from jejunal loop.

Results: Balance



P balance: 10 week old rats had higher positive P balance compared to 20 and 30 week ($p = 0.0009$ *and* $p = 0.0042$). High P diet rats had a higher P balance compared to normal and low ($p = 0.0346$ *and* $p = 0.0322$).

Net P absorption: 10 week old rats had higher positive net P absorption compared to 20 and 30 week. As P in the diet increased, net P absorption was higher ($p < 0.0001$ *for all comparisons*).

Summary/Conclusions

Effect of Age and Diet P Level on Active P Absorption

- P absorption into plasma from the jejunal loop was higher in the 10-week vs 20- and 30-week rats.
- Altering the levels of dietary P did not affect P absorption into plasma.
- P disappearance from the jejunal loop similarly was higher in the 10 week rats, but unchanged by the different diet P levels.

Collectively, these data indicate that younger rats have higher active P absorption efficiency than older rats, but dietary P intake level does not elicit adaptation in active P absorption efficiency after 2 weeks of feeding.

Effect of Age and Diet P Level on P Balance and Net P Absorption

- P balance was higher in the 10-week vs 20- and 30-week rats.
- Balance was higher with the high P diet compared to low and normal P diets.
- Net P absorption was higher in 10-week vs 20- and 30-week rats.
- Net P absorption was also higher with increasing dietary P levels.

Collectively, these data indicate that younger rats have higher P balance and net P absorption compared with older rats, and appear to increase P balance with increasing dietary P more than older rats (though interaction approaching significance), likely reflecting elevated P requirements in growth. All ages of rats had higher net P absorption with increasing dietary P levels, presumably reflecting passive P transport.

Future Directions

- Examine the effect of renal disease progression on intestinal P absorption and transporter expression in the novel Cy/+ rat model of CKD.

References

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Marks J, Debnam ES, Unwin RJ. The role of the gastrointestinal tract in phosphate homeostasis in health and chronic kidney disease. Curr Opin Nephrol Hypertens. 2013;22:481–7.

Appendix F3: ASBMR Annual Meeting 2016, Atlanta, GA

Effect of Age and Dietary Phosphorus Intake on Intestinal Phosphorus Absorption in Male Rats

The purpose of this study was to determine the effects of age and dietary phosphorus intake level on intestinal phosphorus absorption efficiency in male rats. Seventy-two male Sprague Dawley rats, ages 8-weeks, 18-weeks, and ~28-weeks were randomized into defined diets with 0.6% calcium and three different levels of phosphorus: 0.1% (low), 0.6% (normal), or 1.2% (high) for 14 days (n = 8 rats per age and diet group). Rats were housed individually in metabolic cages, and feces, urine, and diet were collected, weighed, and analyzed over the final four days of the study to calculate phosphorus balance. On day 14, phosphorus absorption efficiency was assessed using ligated loops of jejunum. (5 microCi P^{33} in 0.5 mL of transport buffer (0.1 mmol/L phosphate). Serial blood samples were collected over 30 minutes, and the jejunal loop was excised at 30 minutes. Plasma samples and the digested jejunal loop were analyzed for P^{33} activity by scintillation counting. Two-way analysis of variance was used to determine main effects (age and diet) and the interaction effect for age x diet, with Tukey's post-hoc comparisons.

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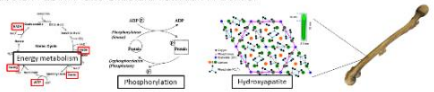

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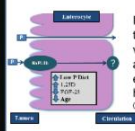

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(Marks J et al. 2013 Curr Opin Nephrol Hypertens.; 22:481)

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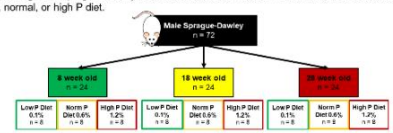
Study design: 72 Sprague-Dawley rats of 8 weeks, 18 weeks, and 28 weeks were randomly assigned a low, normal, or high P diet.



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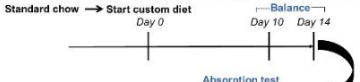

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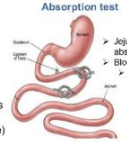

- Jejunal ligated loop absorption test
- Blood collection
  - Baseline, 5, 10, 15, 30 minutes

Sacrifice
- Intestine scraping (jejunum and duodenum) and BBM isolation

% Efficiency: Absorption efficiency was calculated as:
(1-(^{33}P remaining in loop)/Total ^{33}P dose)

Statistics: 2-way ANOVA was performed for all outcomes with the main effects and interaction of diet and age. Post-hoc comparisons were evaluated with the Tukey test.

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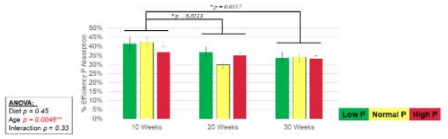

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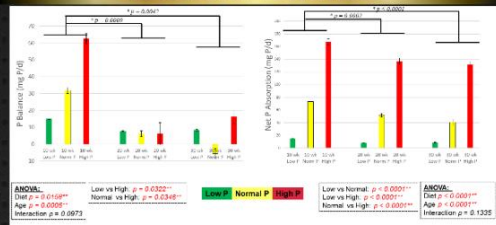

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Appendix F4: Experimental Biology 2017, Chicago, IL

Comparison of Digestion Methods for Phosphorus Analysis of Fecal and Diet Samples

The purpose of this study was to determine fecal and diet phosphrous content comparing four different digestion methods to the standard AOAC perchloric acid method (AOAC). The four methods tested were: muffle furnace at 600 C for 3 days (MF600), muffle furnace at 500 C for 2.5 days (MF500), microwave acid digestion (MICRO), combination muffle furnace and wet digestion (550 C for 1 hour + 70% nitric acid overnight) (MF550+). Homogenized mixed diet samples, NIST wheat flour standard reference material, and homogenized fecal samples were digested by these methods. The mixed diet samples were spiked with 0, 0.45 (low), 0.90 (med), or 1.35 (high) mg phosphorus/gram diet of disodium phosphate, while the fecal samples were spiked with 0, 200 (low), or 400 (high) µg phosphorus in each sample. Phosphorus content of samples digested using the AOAC method was determined by colorimetry, and by ICP-OES for the other four digestion methods. Total phosphorus content and % recovery of spiked samples from the four methods were compared with the AOAC method. For the mixed diet, compared to the AOAC (110 ± 0.9 mg/100 g), MF600 (108 ± 0.9 mg/100g), MF500 (97 ± 2.7 mg/100 g), and MICRO (100 ± 5.2 mg/100 g) but not MF550+ (41 ± 5.2 mg/100 g) yielded similar phosphorus content. Recovery of spiked disodium phosphate on AOAC averaged 103%, MF600 104%, MF500 99%, MICRO 95%, and MF550+ 47%. Bland-Altman plots showing the mean difference between each method and the AOAC and 95% limits of agreement are shown below. Phosphorus content in wheat flour standard on the AOAC method was 1586 ± 36 mg/kg, MF600 was 1526 ± 35 mg/kg, MF500 1623 ± 5 mg/kg, MICRO 1621 ± 10 mg/kg, and MF550+ 1836 ± 32 mg/kg, all higher than the expected of 1333 ± 36 mg/kg. MF600, MF500, and AOAC yielded similar recovery for fecal phosphorus: MF600 2435 ± 196 mg/kg, MF500 2431 ± 108 mg/kg, MICRO 2678 ± 72 mg/kg, MF550+ 2950 ± 71 mg/kg, and AOAC 2428 ± 56 mg/kg. Recovery from fecal phosphorus spiking tended to be overestimated with dry digestion: average for AOAC was 97%, MF600 112%, MF500 111%, MICRO 103%, and MF550+ 100%. Overall, combination dry and wet digestion yielded inconsistent phosphorus content compared to other methods for mixed diet, wheat standard, or fecal samples. Other methods are similar for mixed diet, but all overestimated phosphorus in wheat flour. Spiked recovery for fecal phosphorus was most accurate with microwave, combined dry and wet digestion, and perchloric digestion.

Comparison of Digestion Methods for Phosphorus Analysis of Diet and Fecal Samples

Colby J. Vorland[1], Berdine R. Martin[1], Cheryl Armstrong[1], Scott Radcliffe[2], Ranjani N. Moorthi[3], Sharon Moe[3], Kathleen M. Hill Gallant[1]

[1]Department of Nutrition Science, Purdue University, W. Lafayette, IN. [2]Department of Animal Science, Purdue University, W. Lafayette, IN., [3]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN.

PURDUE UNIVERSITY

Introduction

Phosphorus is ubiquitous in the diet, with the average intake at 1399 mg/d in the U.S. doubling the RDA of 700 mg/d.

Emerging research suggests that consuming too much phosphorus may contribute to an increased risk of heart and bone disease and mortality in healthy adults and in kidney disease. To better characterize these relationships, accurate measurement of phosphorus from diet is necessary. In addition, phosphorus measurement from fecal samples must be precise for balance studies.

The gold standard AOAC method for digestion of feed for analysis of phosphorus utilizes the hazardous chemical perchloric acid. Thus, several studies have examined the combination of heating and other acids with a furnace or microwave against the AOAC method on non-human samples. Such alternative methods are routinely used, but their accuracy on human diet and feces against the AOAC method have not been published. In addition, pure dry digestion without acid has not been evaluated.

Therefore, our **objective** was to compare the measurement and recovery of phosphorus from diet and human fecal samples with several digestion methods compared to the AOAC method.

Methods

Mixed Diet: A meal composite was made by blending the following foods: pork tenderloin, baked potato, sour cream, butter, baby carrots, baguette, applesauce, and skim milk. During blending, it was spiked with either 1) no added P, 2) disodium P, or 3) sodium phytate. P was added at either low (0.45 mg P/g diet), medium (0.90 mg P/g diet), or high (1.35 mg P/g diet) amounts.
Wheat flour standard reference material (SRM): Wheat flour SRM was purchased from NIST (SRM 1567b). Digestion methods included microwave, open digestion, or no digestion (NIST, personal communication). Average P is certified by taking the average of the following analytical methods: DCP-AES, ICP-OES, SPECTRO, WDXRF.
Feces: A fecal homogenate was created by vortexing together and inverting several banked human fecal homogenates from a previous experiment. Fecal samples were spiked with ICP standard (ammonium dihydrogen phosphate) (200 ul or 400 ul) after samples were aliquoted for digestion.

Method Abbreviation	Description	Acid Use for Digestion	Max Temperature [°C]	Total Digestion Time	Analytical Method
MF600	Dry ash (muffle furnace)	-	600	3 days	ICP-OES
MF500	Dry ash (muffle furnace)	-	500	2.5 days	ICP-OES
MICRO	Microwave (CEM Mars 6)	35% HNO_3	210 (diet) 200 (feces)	1 h 20 minutes	ICP-OES
COMB	Dry ash (muffle furnace) + wet digestion (acid)	70% HNO_3	550	1 hour (furnace) Overnight (acid)	ICP-OES
AOAC	Wet digestion ($HClO_4$ + HNO3), AOAC standard	70% HNO_3 + 70% $HClO_4$	200	~4.5 hours	Colorimetric

Method Descriptions
MF600 and MF500: samples were aliquoted into 40 ml crucibles, weighed, and inserted into the muffle furnace (Thermolyne, Thermo Scientific, pictured below). Temperature increased at 5 C/minute and held for 3 days at 600 C (MF600), or 2.5 days at 500 C (MF500). They cooled overnight and were weighed the next morning, and diluted with 2% HNO_3 for analysis on ICP-OES.
MICRO: samples were weighed and aliquoted into vessels, and 5 ml 70% HNO_3, and 5 ml water added and allowed to digest for 30 minutes. Vessels were then sealed and inserted into the microwave (pictured below). The "One Touch" "food" method and a custom "feces" method were used with the following settings, and diluted to 2% HNO_3:
- "Food": ramp time 20:00-25:00, hold time 15:00, temperature 210 C.
- "Feces": ramp time 20:00-25:00, hold time 15:00, temperature 200 C.

COMB: samples were aliquoted into 40 ml crucibles, weighed, and inserted into the muffle furnace. Temperature was ramped to 550 C and held for 1 hour. After cooling (~2 hours), 5 ml 70% HNO_3 was added to samples and heated in a water bath at 70 C for 15 minutes. Samples were then transferred to funnels with #5 filter paper (2.5 µm pore size) for filtration, and diluted with 2% HNO_3 for analysis on ICP-OES.
AOAC: 5 ml 70% HNO_3 was added to aliquoted samples and left overnight. In a $HClO_4$ approved fume hood, 3 ml $HClO_4$ was added, and heated to ~175 C for > 50 minutes, then cooled. Samples were heated to ~200 C for about 3 hours, until white fumes disappeared. Analysis was done using a colorimetric method on a spectrophotometer (PowerWaveX, Bio-Tek Instruments, Inc).

For each method, samples were prepared in triplicate or quadruplicate. Approximately 0.5 g diet and 1.5 g feces were used for all methods.

Bland-Altman plots and 95% limits of agreement were generated with R 3.3.2 with the BlandAltmanLeh package for comparisons between methods. The plots reflect the mean difference between each method and the AOAC method, along with the estimation of the interval that 95% of the differences between the methods fall.

Results

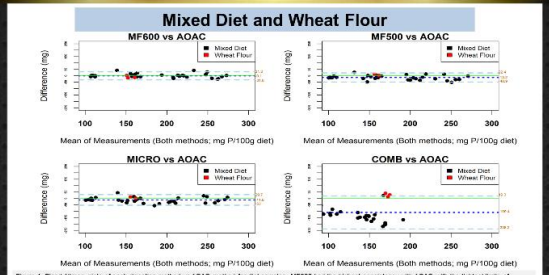

Figure 1. Bland-Altman plots of each digestion method vs AOAC method for diet samples. MF600 had the highest consistency with AOAC with the tightest limits of agreement (mean difference -0.1 mg). The MICRO and MF500 methods had similar consistency (mean difference -13.2 and -11.8 mg). COMB had poor recovery of phosphorus vs AOAC and is not a suitable method for digestion of mixed diet samples. Phosphorus in wheat flour was similar to AOAC with MF600, MF500, and MICRO.

Method	Disodium phosphate	Sodium phytate
MF600	104% (4%)	108% (2%)
MF500	95% (7%)	103% (6%)
MICRO	95% (10%)	104% (7%)
COMB	47% (4%)	56% (5%)
AOAC (+/- SD)	103% (7%)	107% (7%)

Table 1. Recovery of spiked disodium phosphate and sodium phytate for each digestion method. Recovery was approximately 100% for all methods except for COMB, which was poor. Recovery of sodium phytate was consistently higher than disodium phosphate.

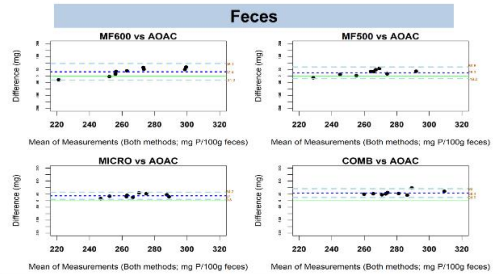

Figure 2. Bland-Altman plots of each digestion method vs AOAC method for human fecal samples. Mean differences for MF600 and MF500 compared to AOAC were similar at 32.4 and 29.3 mg phosphorus. The 95% limits of agreement did not include zero for MICRO or COMB, though mean differences were similar to other methods.

Method	Low Spike	High Spike
MF600	110% (7%)	118% (5%)
MF500	111% (2%)	116% (5%)
MICRO	102% (4%)	104% (2%)
COMB	97% (2%)	102% (6%)
AOAC (+/- SD)	96% (1%)	97% (7%)

Table 2. Recovery of ICP standard spike for each fecal digestion method. MICRO, COMB, and AOAC showed the closest recovery to 100%.

Summary/Conclusions

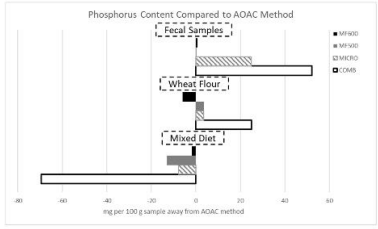

Figure 3. Overall average differences for each digestion method compared for the AOAC method for mixed diet, wheat flour, and fecal samples.

Diet Digestion
- MF600, MICRO, and MF500 are suitable alternatives to the AOAC nitric acid+perchloric acid digestion for measurement of phosphorus in mixed diet samples and wheat flour. Recovery of spiked phosphorus confirmed these results.
- COMB (short dry digestion + nitric acid digestion) is not suitable for mixed diet sample analysis.

Human Fecal Digestion
- MF600 and MF500 are most consistent with the AOAC method for phosphorous measurement in fecal samples. MICRO and COMB slightly overestimated phosphorus but are suitable alternatives. Recovery of spike largely corroborated these results.

Overall conclusions
Muffle furnace at 600 or 500 C, or microwave digestion of diet and human feces are reliable alternatives to the AOAC method. Such methods provide additional choices to the use of the hazardous chemical perchloric acid required by the AOAC method.

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Appendix F5: ASBMR Annual Meeting 2018, Montreal, CA

Effect of Age and Dietary Phosphorus Intake on Phosphorus Regulatory Hormones and Intestinal Phosphate Transporter Gene Expression

We previously reported higher phosphorus (P) absorption efficiency in 10wk SD rats vs older rats and no effect of low P diet on net P absorption using an *in situ* ligated loop method (Vorland JBMR 31(S1)). To understand the mechanisms involved in these age-related changes, we evaluated the effects of age and dietary phosphorus (P) on P-regulating hormones and intestinal P transporter gene expression. N=72 male SD rats studied at 3 ages (10wk, 20wk, and 30wk of age) were fed diets of 0.6% Ca and 0.1% (LP), 0.6% (NP), or 1.2% (HP) P (n=8/group) for 14d before sacrifice. Intestinal P absorption was measured by jejunal ligated loop (5 μCi ^{33}P in 0.5 mL of 0.1 mmol/L P transport buffer) over 30min prior to sacrifice. Jejunal and duodenal mucosal tissue and blood were collected. Gene expression of intestinal P transporters NaPi2b and Pit1 were measured by RT-PCR, plasma 1,25-dihydroxyvitamin D3 (1,25D) by EIA, and intact parathyroid hormone (iPTH), intact and c-terminal fibroblast growth factor 23 (iFGF23, cFGF23) by ELISA. Data were analyzed by 2-way ANOVA for age, diet and interaction effects, with Tukey's post-hoc tests.

1,25D levels were significantly lower, and iFGF23 and iPTH levels were higher at 20 or 30wk compared with 10wk in all animals (all $p<0.05$). LP increased 1,25D, and decreased both iFGF23 and cFGF23 (all $p<0.01$). There was an age x diet interaction only for iPTH ($p<0.0001$) where LP had lower iPTH vs NP and HP, but the magnitude was greatest in 10wk. 10wk rats had higher jejunal NaPi2b mRNA than 20wk and 30wk rats ($p=0.002$ & $p=0.02$), but there was no effect of diet ($p=0.14$), consistent with our previously reported increased P absorption at 10wk versus 20wk and 30wk of age. LP diet increased duodenal NaPi2b mRNA expression, but only in 10wk ($p=0.001$). There were no significant effects for age or diet in Pit1 mRNA in the duodenum ($p=0.50$). In contrast to the decrease in NaPi2b with age, there was an increase in jejunal Pit1 mRNA expression at 30 weeks compared to 10wk and 20wk ($p=0.002$).

These results show that higher P absorption observed in younger normal SD rats is due to higher 1,25D and lower PTH and FGF23, resulting in upregulation of NaPi2b. Overall, LP diet caused expected changes in P-regulating hormones, but only upregulated NaPi2b at 10wk and only in the duodenum, and had no effect on Pit1. This corresponds with the lack of effect of diet P level on intestinal P absorption assessed in the jejunum in these rats.

Effect of age and dietary phosphorus intake on phosphorus regulatory hormones and intestinal phosphate transporter gene expression

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PURDUE UNIVERSITY

Introduction

Phosphorus (P) is an essential nutrient that has critical roles in the body including a structural function in bones as hydroxyapatite and in energy metabolism. Dietary P deficiencies are rare, as P is naturally abundant in the food supply and also added in the form of inorganic P food additives.

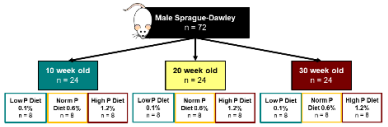
However, there is increasing concern that elevated serum and dietary P increase cardiovascular disease risk and mortality. Intestinal P absorption is an important component to P metabolism, yet factors that influence absorption are understudied. In vitro and ex vivo evidence consistency suggests that the **level of dietary phosphorus** and **age** affect P uptake into the enterocyte. However, absorption in response to these factors has not been adequately tested in a more physiological context.

Our goal is to improve the understanding the capacity for active intestinal P absorption *to adapt* to different factors. Therefore our primary aims were to:

- **Examine the effect of altering the level of dietary P intake and age on in situ intestinal P absorption efficiency, whole-body P balance, phosphorus regulatory hormones, and intestinal phosphate transporter gene expression in healthy rats.** ***We previously reported absorption and balance results, here we additionally report hormones and intestinal phosphate transporter gene expression.***

Methods

Study design: 72 Sprague-Dawley rats were randomly assigned a low, normal, or high P diet at 8 weeks-, 18 weeks-, or 28 weeks-of-age, and P balance and intestinal P absorption assessed after 2 weeks of feeding the assigned diet.

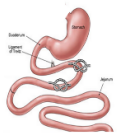


Diet: Rats were placed on the assigned diet for 14 days, while **balance** was done on days 10-14. Calcium was 0.5% on all diets.

Balance: All urine and feces were collected, and diet weighed. Fecal and diet samples were ashed in a muffle furnace. Phosphorus content was measured with ICP-OES.

Absorption test: 5 µCi ^{33}P was injected into a 5cm jejunal loop starting 1cm distal to the ligament of Treitz with 0.5ml uptake buffer (0.1 mmol/L KH_2PO_4). Area under the curve (AUC) was calculated from ^{33}p appearance in plasma at 0, 5, 10, 15, and 30 minute time points. ^{33}P remaining in the loop at 30 minutes was used as the primary absorption measure. Liquid scintillation counting was used to measure ^{33}P activity.

Absorption test



Jejunal ligated loop absorption test
Blood collection
- Baseline, 5, 10, 15, 30 minutes

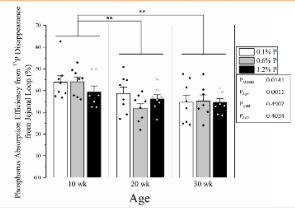
Sacrifice
Intestine scraping (jejunum and duodenum) and BBM isolation for transporter quantification (not shown)

% Efficiency: Absorption efficiency was calculated as: (1-(^{33}P remaining in loop)/Total ^{33}P dose)x100

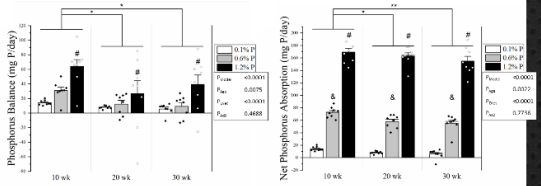
Statistics: Two-way ANOVA was performed for all outcomes with the main effects and interaction of diet and age, with Tukey-adjusted post-hoc pair-wise comparisons.

Results: Absorption & Balance

Absorption and balance results were presented previously at ASBMR 2016

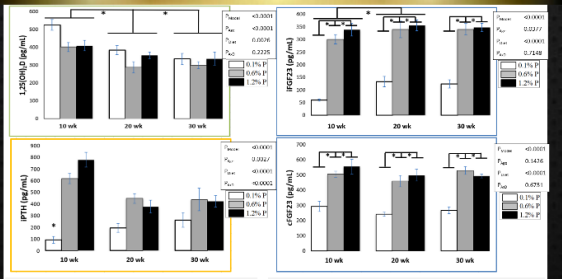


Absorption efficiency from loop: Efficiency was higher in 10 week old rats compared to 20 and 30 (***p < 0.01 for both*), but there were no differences between level of P in the diet (*p = 0.49 for diet main effect*). Both findings were also confirmed when analyzing by AUC of plasma ^{33}P over 30 minutes. No interaction effect was present. Absorption efficiency (%) assessed by disappearance from jejunal loop.



P balance and net P absorption: P balance was higher in 10 week old rats compared to 20 and 30 (**p = 0.01 and p = 0.03*). Balance was also higher with the high P diet (*p < 0.01 vs both NP and LP*). Net P absorption was higher at 10 weeks vs 20 and 30 weeks (*p = 0.047 and p < 0.01 respectively*). There was a stepwise increase in net P absorption with increasing dietary P (*p < 0.0001 for all*).

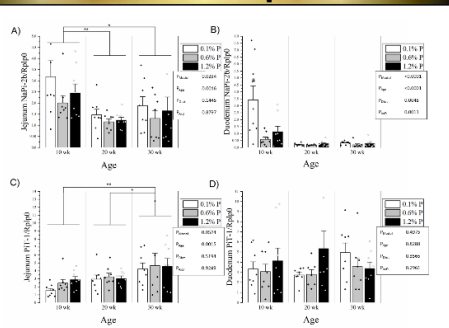
Results: Hormones



1,25D (top): 1,25D was higher at 10 weeks (*p < 0.01 vs both 20 and 30 weeks)*, and higher on LP vs NP (*p < 0.01*).
PTH (bottom): PTH was decreased on LP but the magnitude was greatest at 10 weeks.

iFGF23 and cFGF23: Both were decrease by LP vs NP and HP (*p < 0.0001 for all comparisons*). iFGF23 was lower at 10 weeks vs 20 weeks (*p = 0.049*).

Results: Gene Expression



Jejunal NaPi-2b (A): Gene expression was higher at 10 weeks vs 20 and 30 (*p < 0.01 and p = 0.02*), with no difference between diet groups (*p = 0.14*).

Duodenal NaPi-2b (B): Gene expression was higher at 10 weeks but only in the LP group (*p = 0.001*).

Jejunal PiT-1 (C): Gene expression was higher at 30 weeks, though overall model *p = 0.0524*

Duodenal PiT-1 (D): No difference between groups *(overall model p = 0.50)*

Summary/Conclusions

Effect of Age and Diet P Level on Active P Absorption

- P absorption from the jejunal loop was higher in the 10-week vs 20- and 30-week rats.
- Altering the levels of dietary P did not affect P absorption.

Effect of Age and Diet P Level on P Regulatory Hormones

- 1,25D was higher at 10-weeks vs 20- and 30-weeks, matching absorption results.
- However it was higher on the LP diet, whereas absorption did not increase on LP.
- FGF23 was increased with a NP and HP diet vs LP, reflecting its known suppression on 1,25D

Effect of Age and Diet P Level on P Transporter Gene Expression

- Jejunal NaPi-2b was elevated at 10-weeks but unchanged by dietary P, reflecting absorption patterns.

Collectively, these data indicate that younger rats have higher active P absorption efficiency than older rats, which may be mediated by elevated 1,25D, but dietary P intake level does not elicit adaptation in active P absorption efficiency after 2 weeks of feeding. This is in contrast to the bulk of the in vitro or ex vivo literature and suggests that P absorption should be tested with more physiologic techniques to assess the relevance in the capacity of the intestine to adapt to factors.

Effect of Age and Diet P Level on P Balance and Net P Absorption

- P balance was higher in the 10-week vs 20- and 30-week rats.
- Net P absorption was higher in 10-week vs 20- and 30-week rats.
- Net P absorption was also higher with increasing dietary P levels.

Collectively, these data indicate that younger rats have higher P balance and net P absorption compared with older rats, likely reflecting elevated P requirements in growth. All ages of rats had higher net P absorption with increasing dietary P levels, presumably reflecting passive P transport.

Supported by (NIH) K01DK102864

Appendix F6: ASBMR Annual Meeting 2018, Montreal, CA

Effect of Kidney Disease Progression on Intestinal Phosphorus Absorption in Male Cy/+ Chronic Kidney Disease Rats

The Cy/+ rat has been characterized as a progressive model of chronic kidney disease-mineral bone disorder (CKD-MBD). We aimed to determine the effect of kidney disease progression on intestinal phosphorus (P) absorption and whole-body P balance in this model. N=48 Cy/+ (CKD) and N=48 normal littermates (WT) rats were studied at two ages: 20wk and 30wk, to model early (~50% loss of kidney function) and moderate-late CKD (~75% loss of kidney function), respectively. All rats were placed on a 0.7% Ca, 0.7% P casein-based diet to promote disease progression in the CKD rats. Intestinal P absorption efficiency was measured by ligated loops using ~5 µCi ^{33}P in 0.5mL transport buffer (0.1 mmol/L KH_2PO_4, with or without sodium (Na)) injected into a ~5cm jejunal segment while rats were anesthetized. The 2 different transport buffers assessed Na-dependent and Na-independent components of P absorption (n=12/genotype/age/test). ^{33}P activity was measured in the excised, digested loop by liquid scintillation counting. Absorption was determined by disappearance from the loop at 30min post-injection. P balance was determined over 4d prior to sacrifice. Diet, urine, and fecal P were measured by ICP-OES. Two-way ANOVA was used to determine genotype and age effects and interaction, with Tukey's post-hoc comparisons.

There was a significant genotype x age interaction ($p<0.05$) for Na-dependent absorption with decreased absorption from 20wk to 30wk in the CKD rats (13 to 5%, $p < 0.01$) and no difference in WT rats. However, there were no differences for genotype, age, or interaction observed for Na-independent absorption efficiency (overall p=0.36). CKD rats had marginally lower P balance compared to WT (-10 vs -2 mg/day, p=0.06). There was a significant effect of age where 30wk rats had lower P balance compared to 20wk rats, regardless of genotype (-13 vs 1 mg/day, $p<0.001$). There was no significant genotype x age interaction.

These results demonstrate decreasing Na-dependent P absorption with progression of CKD when assessed by an *in situ* ligated loop test.

Effect of kidney disease progression on intestinal phosphorus absorption in male Cy/+ rats

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PURDUE UNIVERSITY

Background

Chronic kidney disease (CKD) affects more than 10% of the U.S. population and virtually all CKD patients develop features of CKD-mineral bone disorder (CKD-MBD). CKD-MBD is characterized by biochemical abnormalities related to mineral metabolism and clinical consequences of increased cardiovascular disease, bone fragility fracture, and related mortality (Figure 1).

Abnormal phosphorus homeostasis is considered a central factor in the development of CKD-MBD. Treatment of CKD-MBD in part includes limiting the intestinal absorption of dietary P though the regulation of intestinal P in CKD is not well understood. Abnormal P metabolism (FGF-23, 1,25D, PTH) occurs in early stages of CKD, yet most research focuses on late stage CKD (Figure 2). It is unclear how CKD progression affects intestinal absorption efficiency. In addition, whether sodium-independent transport changes in CKD has not been assessed in vivo.

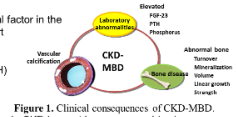


Figure 1. Clinical consequences of CKD-MBD.

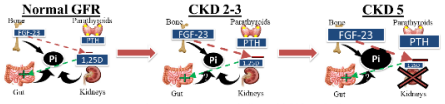


Figure 2. Progressive changes in P metabolism with kidney decline.

Most animal models of CKD have used surgical or chemical methods in Sprague-Dawley rats to induce late stage kidney disease. The Cy/+ rat has a Sprague-Dawley background and a spontaneous genetic mutation that produces a progressive CKD phenotype, which allows us to study how intestinal P absorption is regulated at early and late stages of disease. The ligated loop absorption test allows us to test this in vivo where most studies use in vitro techniques. In addition, the relative contributions of both sodium-dependent and sodium-independent transport can be measured using uptake buffers with and without sodium.

An overarching goal of our lab is to improve the understanding of how active intestinal P absorption *adapts* to different factors. Our primary aim for the current study is to:

- **Examine the effect of kidney function decline on intestinal phosphorus absorption efficiency using the in situ ligated loop absorption test.**

Methods

Study design: 96 male Cy/+ rats (Hannover Sprague-Dawley background) were randomly assigned to the 20 or 30 week age group, and within these groups randomly assigned to a sodium-dependent or sodium-independent uptake buffer group (Figure 4).

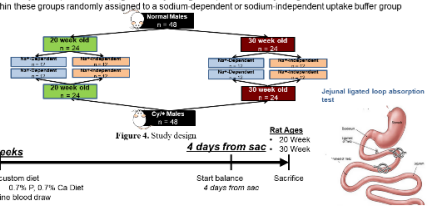


Figure 4. Study design

Timeline:

Diet: All rats are placed on a 0.7% P casein-based diet at 16 weeks to trigger kidney disease progression.

Transporter analysis: The mucosal layer of the intestine was scraped of the jejunum and duodenum immediately at sacrifice, and snap-frozen for RNA analysis.

Plasma measures: Plasma P, Ca, and creatinine were measured from plasma using COBAS. Hormones involved in the homeostatic regulation of phosphate, FGF-23, PTH, and 1,25D, were measured from plasma by ELISA kits.

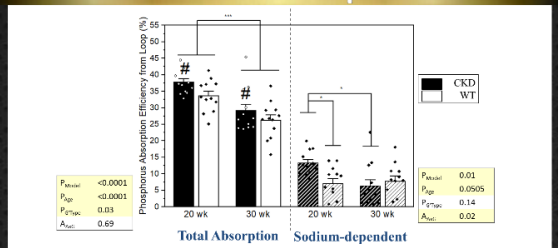
Absorption efficiency (%) is calculated as: (1-(^{32}P remaining in loop)/Total ^{32}P dose)x100

Balance: All urine and feces are collected, and diet weighed daily for 4 days. Fecal and diet samples are ashed in a muffle furnace. Phosphorus content is measured with ICP-OES.

Absorption test: 5 μCi ^{32}P is injected into a 5cm jejunal loop starting 1cm distal to the ligament of Treitz with 0.5 ml uptake buffer (0.1 mmol/l. KH_2PO_4, with or without sodium) while rats are anesthetized. Area under the curve (AUC) is calculated from ^{32}P appearance in plasma at 0, 5, 10, 15, and 30 minute time points. ^{32}P remaining in the loop at 30 minutes is also used to quantify absorption. Liquid scintillation counting is used to measure ^{32}P activity.

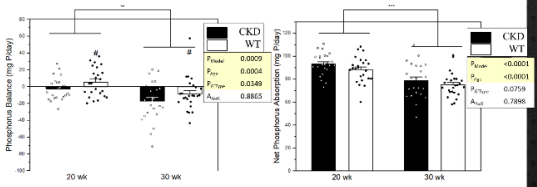
Statistics: 2-way ANOVA was be performed for all outcomes with the main effects and interaction of age and genotype, with Tukey-adjusted post-hoc pair-wise comparisons.

Results: Absorption



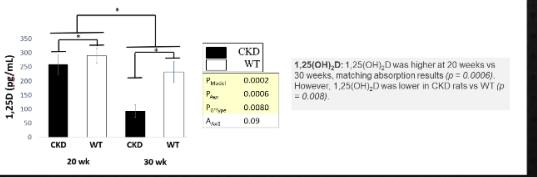
Jejunal phosphorus absorption efficiency: Total absorption was higher at 20 weeks vs 30 ($p < 0.0001$), and higher in CKD rats vs WT ($p = 0.03$). When subtracting transport after giving the sodium-free absorption buffer, sodium-dependent transport was higher in 20-week CKD rats vs 20-week WT and 30-week CKD rats ($p = 0.02$ and $p = 0.0003$).

Results: Balance



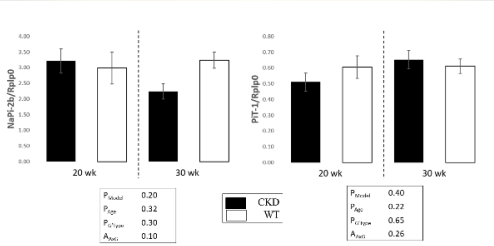
Phosphorus balance and net phosphorus absorption: phosphorus balance was lower at 30 weeks vs 20 weeks ($p = 0.0004$), and lower in CKD rats vs WT ($p = 0.03$). Net phosphorus absorption was lower at 30 weeks vs 20 weeks, matching the ligated loop results ($p < 0.0001$).

Results: 1,25(OH)$_2$D



1,25(OH)$_2$D: 1,25(OH)$_2$D was higher at 20 weeks vs 30 weeks, matching absorption results ($p = 0.0006$). However, 1,25(OH)$_2$D was lower in CKD rats vs WT ($p = 0.008$).

Results: Jejunal NaPi-2b & PiT-1



Jejunal NaPi-2b and PiT-1 gene expression: No differences by age or genotype were observed (NaPi-2b, p = 0.20; PiT-1, p = 0.40)

Summary

- Phosphorus absorption was decreased at 30 weeks vs 20 weeks as assessed by the ligated loop absorption test and net phosphorus absorption from metabolic balance. This is in contrast to our previous study in healthy commercial Sprague Dawley rats, but corresponds to a decreased 1,25D at 30 weeks.
- In contrast to our hypothesis, CKD rats had higher absorption as measured by the ligated loop. This is despite the decreased 1,25D in CKD rats.
- Phosphorus balance was decreased at 30 weeks and in CKD rats. This may reflect bone loss in CKD.
- There were no differences in NaPi-2b or PiT-1 gene expression in the jejunum.

The lack of decrease in phosphorus absorption in CKD does not follow the decrease in 1,25D with disease. This suggests that 1,25D is not a strong regulator of absorption outside of deficiency or additional regulators exist to maintain intestinal phosphorus absorption in CKD.

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Supported by (NIH) K01DK102864 and the Showalter Research Trust

Appendix F7: The American Society for Nephrology (Kidney Week) Annual Meeting 2018, Chicago, IL

Effect of ovariectomy on the progression of chronic kidney disease-mineral bone disorder (CKD-MBD) in Cy/+ rats

Background:

There is increasing interest in sex as a biologic variable, yet studies have generally not examined the role of sex in the pathogenesis of CKD-MBD despite experimental and epidemiological evidence suggesting that estrogen is protective to kidney function and bone and thus CKD-MBD. In the Cy/+ rat model of CKD-MBD, a spontaneous genetic mutation causes progressive kidney function decline in males prior to 20 weeks of age, but kidney function is maintained in females past 80 weeks of age making it impractical to study these females as a model of CKD. Therefore, ovariectomy to mimic a post-menopausal state may accelerate the initiation of the CKD-MBD phenotype and enable the use of female Cy/+ rats in research. The primary aim of this study was to determine if ovariectomy in Cy/+ females would cause kidney function decline more similarly to Cy/+ males.

Methods:

Sixteen female Cy/+ rats were randomized to either ovariectomy (OVX) (n=8) or sham surgery (n=8) at 15 weeks of age. A casein-based diet was initiated at 24 weeks of age to promote kidney function decline as is done in studies with male Cy/+ rats. Blood was sampled at 10, 20, 25, 30, and 35 weeks of age, and analyzed for BUN, plasma phosphorus, and plasma calcium.

Results:

Data collected on all n=16 through 25 weeks show that OVX rats have higher body weights ($p<0.0001$) (and lower uterine weights for n=4 that completed the 35 weeks of the study) confirming the success of the OVX procedure. Plasma phosphorus decreased over time in both groups ($p<0.0001$), but was not different between groups ($p=0.46$). Plasma calcium was not different between groups ($p=0.38$) and did not change over time ($p=0.57$). Plasma BUN decreased slightly over time in both groups ($p<0.01$) but remained in normal ranges, and there is no difference between OVX and sham ($p=0.23$). In n=2 OVX and n=2 sham that have completed the 35 weeks of the study, preliminary analysis shows no appreciable difference in BUN, phosphorus, or calcium between groups.

Conclusion:

Analyses will continue through 35 weeks, however at 25 weeks of age, there is currently no indication that OVX accelerates kidney function decline in female Cy/+ rats. This is in contrast to Cy/+ male rats which can be phenotyped based on elevated BUN as early as 10 weeks of age, and by 25 weeks of age exhibit a ~50% reduction in kidney function (Moe et al. 2011).

Effect of ovariectomy on the progression of chronic kidney disease-mineral bone disorder (CKD-MBD) in Cy/+ rats

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PURDUE UNIVERSITY

Background

Chronic kidney disease (CKD) affects more than 10% of the U.S. population and virtually all CKD patients develop features of CKD-mineral bone disorder (CKD-MBD). CKD-MBD is characterized by biochemical abnormalities related to mineral metabolism and clinical consequences of increased cardiovascular disease, bone fragility fracture, and related mortality (Figure 1).

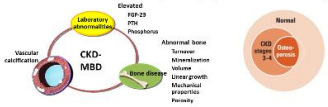

Figure 1. Clinical consequences of CKD-MBD.

Abnormal mineral metabolism (phosphorus and calcium along with regulatory hormones FGF-23, 1,25D, and PTH) occurs in early stages of CKD, yet most research focuses on late stage CKD (Figure 2). **Many research questions that probe disease prevention remain to be answered.**

Further, most animal studies on CKD utilize male rats, potentially overlooking important biological differences in half of the population. For example, in one of our lab's area of interest, intestinal phosphorus absorption, **only 2 of 29 studies have included females** (Figure 2), despite research showing a direct effect of estrogen on the main intestinal phosphate transporter, strong evidence that estrogen impacts bone and mineral metabolism, and experimental and epidemiological evidence suggesting estrogen is renal protective.

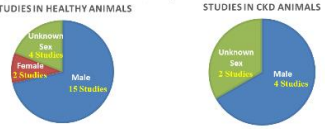

Figure 2. The disproportionate representation of female animals in intestinal phosphate research.

Most animal models of CKD have used surgical or chemical methods in Sprague-Dawley rats to induce kidney disease. The **Cy/+ rat** has a Sprague-Dawley background and a spontaneous genetic mutation that produces a progressive CKD phenotype, which allows us to study how intestinal P absorption is regulated at early *and* late stages of disease.

Cy/+ female rats do not experience kidney decline until after 40 weeks of age, whereas males begin before 20 weeks, so females have been excluded in studies in this model of CKD-MBD thus far. Given the potential renal protective nature of estrogen, we hypothesize that ovariectomy of female Cy/+ rats will produce an accelerated CKD-MBD phenotype and allow for their use in research studies.

Methods

Study design: 16 female Cy/+ rats were randomly assigned to the receive ovariectomy (OVX) (n=8) or sham surgery (n=8) at 15 weeks of age. Baseline blood was taken at 10 weeks of age, and additional blood draws were taken at 20, 25, 30, and 35 weeks of age. At 35 weeks of age, rats were sacrificed and bones, kidneys, and aorta were collected (Figure 3).

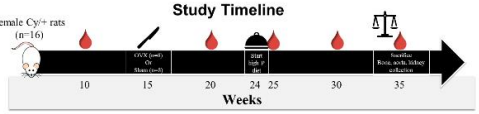

Figure 3. Timeline of surgeries, blood draws, and tissue collections.

Surgery: At 15 weeks of age, rats undergoing OVX had both ovaries surgically removed under anesthesia/Buprenex, and for Sham surgery all incisions were made except for removal of the ovaries.

Diet: All rats were placed on a 0.7% P casein-based diet at 24 weeks to enhance kidney disease progression, as used in previous studies in males.

Metabolic Balance: All urine and feces were collected and diet weighed daily in the 34th week of age in metabolic cages for 4 consecutive days. Fecal and diet samples were ashed in a muffle furnace. Phosphorus content was measured with ICP-OES.

Plasma BUN, creatinine, phosphorus, and calcium, and microCT analysis on tibias were analyzed in rats to test the hypothesis that ovariectomy accelerates kidney disease and abnormal mineral homeostasis. Repeated measures ANOVA was performed on plasma measures and body weight, and t-test on kidney and uterine weights, and microCT and balance measures.

Results: Weights

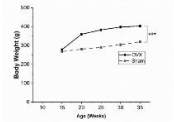

Figure 4. Bodyweight after OVX or Sham surgery over time. Bodyweight was higher in OVX rats vs Sham. *** p<0.0001 between groups.

	OVX (n = 8)	Sham (n = 8)	P
Kidney weight (g)	1.417 ± 0.075	1.382 ± 0.050	0.70
Uterine weight (g)	0.16 ± 0.01	0.78 ± 0.06	< 0.0001

Table 1. Kidney and uterine weight at 35 weeks of age. Kidney weight was not different between groups. Uterine weight was lower in OVX rats.

Results: MicroCT

	OVX (n = 8)	Sham (n = 8)	P
BV/TV (%)	0.99 ± 0.34	17.45 ± 1.59	< 0.0001
Tb.N (mm^{-1})	0.14 ± 0.04	2.16 ± 0.13	< 0.0001
Tb.Th (mm)	0.07 ± 0.008	0.08 ± 0.003	0.15
Tb.Sp (mm^{-1})	0.84 ± 0.02	0.27 ± 0.01	< 0.0001

Table 2. Microstructural parameters of cancellous bone of the tibia measured by micro-CT. BV/TV (bone volume: (BV) fraction (TV = tissue volume)); Tb.N (trabecular number); Tb.Th (trabecular thickness); Tb.Sp (trabecular separation). **No cortical porosity was noted for any animal.**

Results: Balance

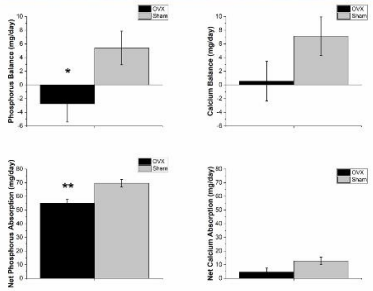

Figure 5. Mineral balance and net mineral absorption at 35 weeks by OVX or Sham surgery. Top left: phosphorus balance was lower in OVX rats vs Sham ($p=0.04$). Top right: calcium balance was not different between the groups ($p=0.12$). Bottom left: net phosphorus absorption was lower in OVX rats vs Sham ($p=0.002$). Bottom right: net calcium absorption was not different between groups ($p=0.07$). * $p<0.05$, ** $p<0.01$.

Results: Kidney Function Biochemistries

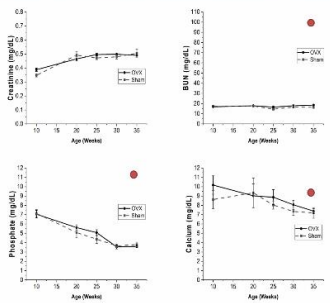

Figure 6. Plasma biochemistries over time between OVX and Sham surgery. Plasma creatinine ($p=0.43$), BUN ($p=0.09$), phosphate ($p=0.61$), and calcium ($p=0.39$) were not different between the groups.

● = Cy/+ male rats at 34 weeks *(data from Moe et al. 2009 Kidney Int)*

	OVX (n = 8)	Sham (n = 8)	P
Creatinine clearance (mL/min)	4.2 ± 0.2	4.1 ± 0.2	0.83

Table 3. Creatinine clearance at 35 weeks of age. OVX and Sham groups were not different.

Summary/Conclusion

- Ovariectomy in Cy/+ rats resulted in decreased uterine weight and expected changes in bone microstructure, and negative phosphorus balance.
- However, plasma markers of kidney function, creatinine, BUN, phosphate, and calcium did not change between groups from the time of ovariectomy at 15 weeks until 35 weeks of age.
- Ovariectomy of the Cy/+ rat is not a suitable method to create a female CKD or CKD-MBD phenotype for research purposes.

References

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Moe, S. et al. A Rat Model of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD) and The Effect of Dietary Protein Source. 2009;75(2):176-184.

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VITA

Education

Ph.D. in Nutrition Science, 2019 (expected)
Interdepartmental Nutrition Graduate Program
Purdue University, *West Lafayette, IN*
Dissertation: The Physiological Relevance of the Adaptive Capacity of Intestinal Phosphorus Absorption

M.Sc in Food and Nutritional Science, 2013
Concentration in Human Nutrition
University of Wisconsin-Stout*, Menomonie, WI*
Thesis: Contribution of Dietary Fat to High-Density Lipoprotein Triacylglycerol in an Overnight Period in Non-alcoholic Fatty Liver Disease

B.Sc in Human Biology with a Nutrition emphasis, 2009
Didactic Program in Dietetics, verification statement earned
Minor in Spanish
University of Wisconsin-Green Bay*, Green Bay, Wisconsin*

Research Experience

Graduate Research Assistant, *Purdue University,* 2013-2018
Conducted laboratory research under the mentorship of Dr. Katie Hill Gallant at Purdue under a graduate assistantship. My dissertation focuses on the adaptive capacity of intestinal phosphorus absorption in health and chronic kidney disease and will result in four first author publications.

- Developed a protocol to use an in situ intestinal phosphorus absorption method in rats.
- Used the absorption method to characterize absorption changes in response to age, dietary phosphorus level, and kidney disease.
- Analyzed human and rat diet, urine, and fecal samples for mineral content to assess phosphorus balance in response to those factors.

- Helped to initiate and now manage a rat breeding colony to study progressive kidney disease.

Graduate Research Assistant, *University of Wisconsin-Stout,* 2011-2013
Performed research under the mentorship of Dr. Kerry Peterson at UW-Stout under a graduate assistant grant. The work was for my master's thesis, isolating HDL from non-alcoholic fatty liver disease patients over nocturnal time-points to quantify HDL concentration and isotopically labeled dietary triglyceride transfer to HDL.

- Experience with Folch extraction, thin layer chromatography, and GC-MS

Undergraduate Research, *University of Wisconsin-Green Bay*, 2009-2010
Assisted in a study on the effects of vitamin D on athletic performance in athletes at UW-Green Bay in '09-'10. Involved in the study idea conception and literature reviewing, implementation, recruiting, and testing. The principal investigator was Dr. Debra Pearson.

Publications

Peer-Reviewed Original Research

- **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. https://doi.org/10.1371/journal.pone.0207601
- **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* (2018) 2(2): 103. https://doi.org/10.1002/jbm4.10026.

Peer-Reviewed Review Articles

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

- Moorthi RN, **Vorland CJ,** & Hill Gallant, KM. Diet and Diabetic Kidney Disease: Plant Versus Animal Protein. *Curr Diab Rep* (2017) 17: 15. https://doi.org/10.1007/s11892-017-0843-x

In Preparation

- **Vorland CJ**, Lachcik PJ, Biruete A, Chen NX, Moe SM, & Hill Gallant, KM. Effect of Kidney Disease Progression on Intestinal Phosphorus Absorption and Phosphorus Balance in Male Rats.
- **Vorland CJ**, Lachcik PJ, Swallow E, Metzger C, Allen M, Chen NX, Moe SM, & Hill Gallant, KM. Effect of Estrogen Deficiency on the Progression of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD) in Cy/+ Rats.

Abstracts and Presentations for Scientific Meetings

- **Vorland, CJ.**, Aromeh, L., Lachcik, PJ., Moe, SM., Chen, NX., Hill Gallant, KM. Effect of Age and Dietary Phosphorus Intake on Phosphorus Regulatory Hormones and Intestinal Phosphate Transporter Gene Expression. Presented at The American Society for Bone and Mineral Research meeting, Montreal, Canada, September 2018.
- **Vorland, CJ.**, Lachcik, PJ., Moe, SM., Chen, NX., Hill Gallant, KM. Effect of Kidney Disease Progression on Intestinal Phosphorus Absorption in Male Cy/+ Chronic Kidney Disease Rats. Presented at The American Society for Bone and Mineral Research meeting, Montreal, Canada, September 2018.
- **Vorland, CJ.**, Lachcik, PJ., Nelson, C., Chen, NX., Hill Gallant, KM. Effect of Ovariectomy on the Progression of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD) in Cy/+ Rats. Presented at The American Society for Nephrology (Kidney Week) meeting, San Diego, CA, October 2018.
- **Vorland, CJ.**, Martin, BR., Armstrong, C., Radcliffe, S., Moe, SM., Hill Gallant, KM. Comparison of Digestion Methods for Phosphorus Analysis of Fecal and Diet Samples. Presented at the Experimental Biology meeting, Chicago, IL, April 2017.
- **Vorland, CJ.**, Lachcik, PJ., Fleet, JC., Hill Gallant, KM. Effect of Age and Dietary Phosphorus Intake on Intestinal Phosphorus Absorption in Male Rats. Presented at The

American Society for Bone and Mineral Research meeting, Atlanta, GA, September 2016.

- **Vorland, CJ.**, Lachcik, PJ., Fleet, JC., Hill Gallant, KM. Effect of Age and Dietary Phosphorus Intake on Intestinal Phosphorus Absorption in Male Rats. Presented at The Indiana Musculoskeletal Symposium, Indianapolis, IN, June 2016.
- **Vorland, CJ.**, Martin, BR., Weaver, CM., Peacock, M., Hill Gallant, KM. Effect of Dietary Calcium on Phosphorus Balance and Net Absorption in Healthy Adolescent Girls. Presented at The American Society for Bone and Mineral Research meeting, Houston, TX, September 2014.
- **Vorland, CJ.**, Peterson, KD. Contribution of Dietary Fat to High-Density Lipoprotein Triglyceride During Nocturnal Hypertriglyceridemia. Presented at the Experimental Biology meeting, San Diego, CA, April 2014.

Teaching Experience

- **Graduate Teaching Certificate**, Purdue University Center for Instructional Excellence, completed 2017
    - Consisted of attending teaching workshops, teaching observations, incorporating student feedback.
- Graduate Teaching Assistant for NUTR 424 Nutrition Communication, Purdue University, Spring 2014-Fall 2018 (7 semesters)
    - Led labs, graded presentations and assignments, assisted with classroom activities, lectured. Varying 25-50 students.
    - Lead instructor for the course in Spring 2017.
- Graduate Teaching Assistant for NUTR 453 Food Chemistry lab section, Purdue University, Fall 2013 (1 semester)
    - Taught 3-hour weekly lab of 24 students.
- Graduate Teaching Assistant for undergraduate Human Nutrition course, 2008, University of Wisconsin-Green Bay (1 semester)

Invited Guest Lectures

- NUTR 480 Medical Nutrition Therapy I, Purdue University, "Cognitive Biases and Critically Evaluating Research", Fall 2014, 2015, 2016, 2017, 2018
- NUTR 424 Nutrition Communication, Purdue University, "Professional Benefits of Social Media", 2017

Other Science Communications

- American Society for Nutrition – Official Meeting Blogger for Experimental Biology, 2014, 2017
- American Society for Nutrition – Official Student Blogger, 2013-2014, 2017-2018
- Article about the Dietary Guidelines co-written with Dr. Connie Weaver for the Indiana State Department of Health Food Protection Program "FoodBytes" Winter 2016 newsletter: https://www.in.gov/isdh/files/FoodbytesWinter_2016.pdf
- Creator and blogger of a nutrition science blog, nutsci.org, 2009-present

Scientific Tools and Software Developed

- **Creator of LazyScholar.org**: a browser extension that finds full scholarly article texts, metrics, more.
- **Creator of nutsci.com**: aggregates new nutrition papers into a sortable table.
- **Creator of ishouldbewriting.net**: tool to encourage writing.
- Additional projects listed at http://nutsci.org/projects

Memberships and Affiliations

- The American Society for Nutrition *2012-present*
- The American Society for Bone and Mineral Research *2014-present*
- The American Society for Nephrology *2016-present*
- The Academy of Nutrition and Dietetics *2007-2009, 2017-present*

Honors and Awards

- Endocrine Fellows Foundation Forum travel grant, American Society for Bone and Mineral Research/The Endocrine Society, Fall 2016