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Role of Sodium/Hydrogen Exchanger Isoform NHE3 in Gastrointestinal Phosphate Absorption

Dissertation

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Table of Contents

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List of Abbreviations

ACM	Acid Microclimate
AstraNIhn	AstraZeneca NHE3 Inhibitor
BBM	Brush Border Membrane
BBMV	BBM Vesicle
Ca / Ca^{+2}	Calcium / Calcium ion
CKD	Chronic Kidney Disease
CKD-MBD	CKD Mineral Bone Disorder
CFTR	Cystic Fibrosis Transmembrane Regulator
COX-2	Cyclo-oxygenase isoform 2
cRNA	copy Ribo-Nucleic Acid
DMSO	Dimethyl Sulfoxide
DRA	SLC26A3 Cl^-/HCO_3^- exchange transport protein
ENaC	Epithelial Na^+ Channel
ESRD	End Stage Renal Disease
FGF 23	Fibroblast Growth Factor 23
GI	Gastro Intestinal
Gly-Sar	Glycyl-Sarcosine
IKEPP	Intestinal and Kidney Enriched PDZ domain Protein
I.D.	Internal Diameter
i.p.	Intra-peritoneal
Isc	Short-circuit current
IU	International Unit
KO	Knock Out
MEPE	Matrix Extracellular Phosphoglycoprotein
NaPi IIb	Na^+ Pi co-transporter isoform IIb
NHE3	Na^+/H^+ Exchanger isoform 3
NHERF	Na^+/H^+ Exchanger Regulatory Factor
PAT-1	SLC26A6 Cl^-/HCO_3^- exchange transport protein
PDZK1	PDZ domain binding Kinase 1
PEG	Polyethylene Glycol
PepT1	Peptide Transporter isoform 1
PFA	Phosphonoformic Acid
Pi	inorganic Phosphate
PiT	Phosphate Transporter
PTH	Parathyroid Hormone
S1611	a selective NHE3 inhibitor drug
s.c.	subcutaneous
sFRP4	secreted Frizzled Related Protein 4
SGLT1	Sodium Glucose Transporter isoform 1
TJ	Tight Junction
WT	Wild Type
ZTL	Zentrales Tierlabor der Medizinischen Hochschule Hannover

List of Figures

1. Introduction

1.1. General Considerations

Chronic Kidney Disease (CKD) describes a gradual loss of renal function, usually leading to an End Stage Renal Disease (ESRD). Diabetes is the most common cause of CKD, and together with hypertension and glomerulonephritis, comprises as a cause for 75% of cases. Most cases of CKD develop slowly and the symptoms may not be apparent until late stages. The most common complications are the direct consequences of a failing kidney function and result in fluid overload, electrolyte imbalances, anemia and irreversible kidney damage. A failing kidney can also lead to cardiovascular complications, infections and bone disorders.[1] Cardiovascular complications constitute the biggest independent cause of mortality in CKD, and have an alleged role in increased morbidity and mortality irrespective of the CKD progression.[1] Bone disorders, also known in CKD consortium as Mineral Bone Disorder (CKD-MBD), include abnormalities of bone growth, bone loss, parathyroid dysfunction and vascular and soft tissue calcification.[2] CKD-MBD and cardiovascular disorders in CKD are hence, in a way, a spectrum of the same disease and have been implicated as direct and indirect consequence of defective metabolism of phosphate (see Figure 1).[3] As much as so that the derangement of phosphate metabolism is considered as an important prognostic factor, and prevention and treatment thereof, as an important therapeutic target in CKD treatment.[3]

Mineral phosphate is freely filtered with the glomerular filtrate and the kidney serves to reabsorb most of the filtered phosphate. But, for maintenance of normal phosphate homeostasis, it is important for the kidneys to excrete some phosphate and maintain normo-phosphatemia. A failing kidney is unable to excrete enough phosphate and is associated with a progressive hyperphosphatemia. The body tries to counteract this by decreasing the reabsorption of phosphate in functional nephrons and by modulating various hormonal and non-hormonal regulators.[3] In the early stages, the failing kidneys are able to completely normalize blood phosphate levels, and this can be mistaken as normophosphatemia. But, early recognition and control of this positive imbalance is important to prevent the morbidity and mortality associated with CKD and has also been suggested to slow the progression of CKD itself.[3] A better understanding of phosphate dysregulation in CKD requires an understanding of normal phosphate homeostasis.

1.2. Phosphate Homeostasis

Phosphate is a major mineral in vertebrate body and is present in organic and inorganic

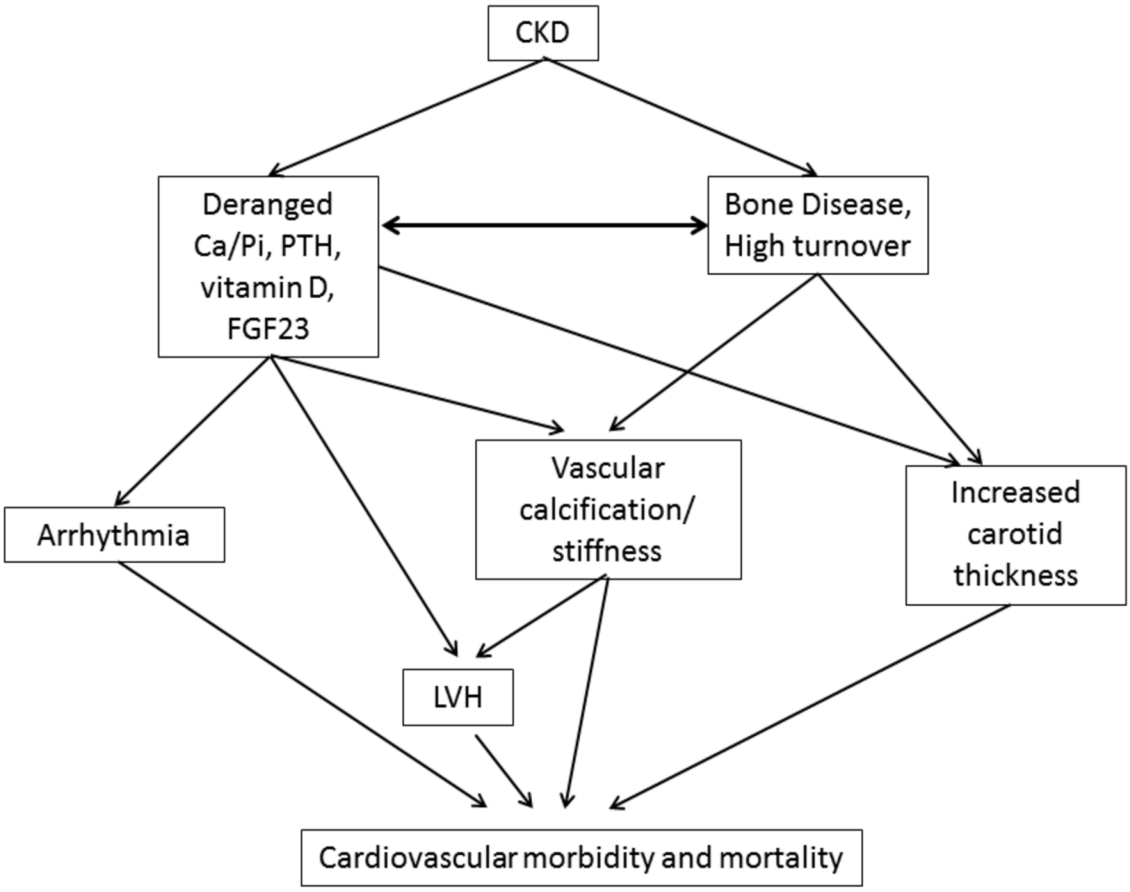


Figure 1 : Cardiovascular and Bone disease in CKD.

(Modified from Eddington *et al.* 2010)

forms. As organic phosphate, it is important component of DNA, RNA, proteins and phospholipids, and serves both as a structural and as a regulatory component. Inorganic phosphate (Pi), present in forms of dihydrogen ($H_2PO_4^-$) and mono-hydrogen (HPO_4^{2-}) anions, serves as a source for the organic phosphate, and also has its independent roles. It has important functions in bone growth and stability, maintenance of pH as a major buffer system, cell signaling and energy metabolism.[4] Inorganic phosphate is the functionally major form of phosphate in the body. Bones serve as the largest storage pool, containing 85% of total body phosphate. Soft tissues contain 14% and extracellular fluid compartment has only 1% of the remaining phosphate. Pi has limited solubility in the presence of divalent cations and it is this form of phosphate whose concentration is more actively regulated in both extra-cellular and intra-cellular fluid compartments.[1] Such tight regulation is also necessary for the concentration of serum calcium (Ca), which is regulated together with Pi by many common regulatory factors. While no exact solubility product for Ca and Pi is known, Ca concentration is lowered with increasing Pi and *vice versa*.

Dysregulation of inorganic phosphate can be characterized as hypo- or hyperphosphatemia. Hypophosphatemia presents less commonly and is associated with disorders of phosphate absorption from the intestinal tract in malabsorption and malnutrition, and disorders of renal reabsorption of filtered phosphate in hypophosphatemic rickets, X-linked hypophosphatemia and tumor induced osteomalacia.[5] Chronic hypophosphatemia is even less common but can lead to bone demineralization. Hyperphosphatemia is more commonly seen and is mostly associated with CKD, which itself has a rising incidence with increased longevity and higher prevalence of diabetes. Hyperphosphatemia in the setting of CKD can lead to secondary hyperparathyroidism and its associated complications[4], which in turn accelerates its progression to ESRD.[6] It has been identified to be an important factor for endothelial dysfunction, cardiovascular morbidity and mortality.[7]

Pi homeostasis requires maintenance of both total body Pi and concentration of serum Pi. Pi enters the body through the intestinal tract and exits with the renal filtrate. With a neutral Pi balance, the daily Pi absorption (approximately 1 – 1.5 g/24h) is equivalent to the total Pi excretion.[5] Phosphate transport in the intestinal tract and in renal filtrate are regulated at cellular level by control of its absorption in enterocytes and its reabsorption in the renal epithelium (proximal convoluted tubule and proximal straight tubule). Both these processes are also important for regulation of the serum Pi concentration, which is otherwise also affected by the transport of Pi between extracellular and intracellular compartments. The skeletal system serves important roles with the latter and acts like a buffer for both serum Ca and Pi concentration (see Figure 2). Vitamin D is an important regulator of intestinal Pi absorption, and Parathyroid Hormone (PTH) is one of the more important regulators of its renal reabsorption. With low Pi diet, the serum Pi concentration falls and leads to an increased hydroxylation of 25-hydroxy vitamin D; the product (1,25-dihydroxy vitamin D) in turn increases Pi and Ca absorption in the enterocytes. Ca concentration in the serum is increased and in turn decreases PTH secretion from the parathyroid glands. PTH is an

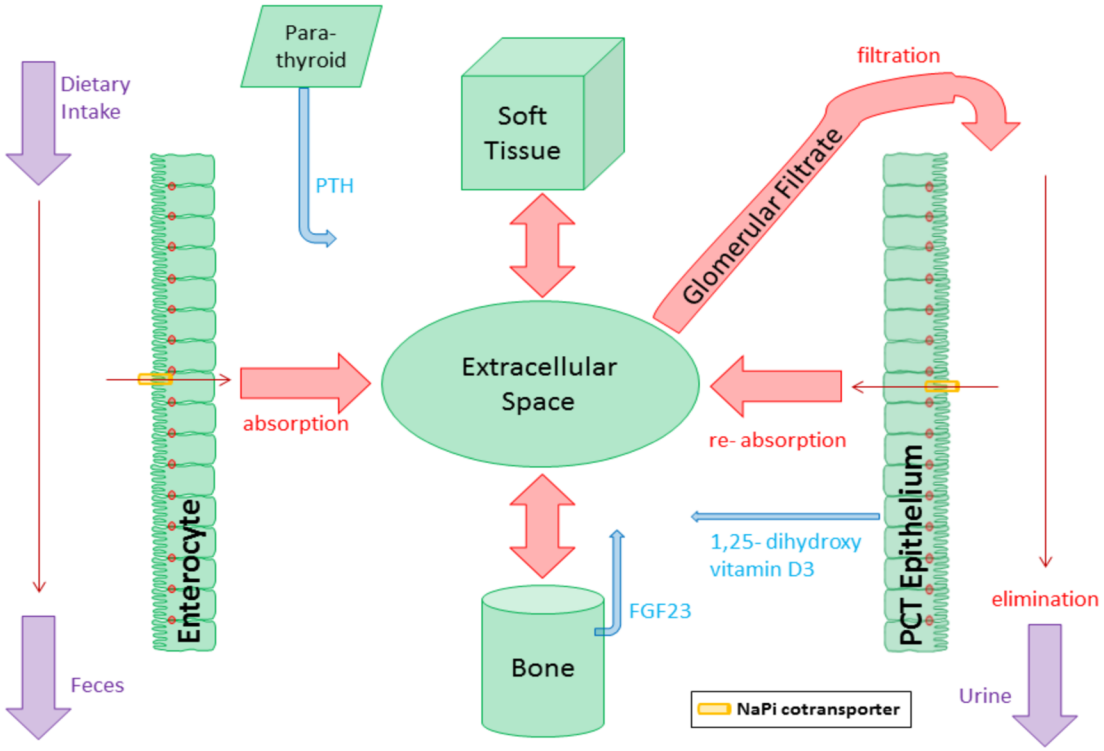


Figure 2: Cartoon representation of phosphate homeostasis in the body.

The amount of phosphate is regulated at the level of three different processes: 1) absorption from the intestinal tract, 2) reabsorption from the renal filtrate, and 3) exchange with the bone storage pool. Different regulatory factors/hormones are involved in this homeostasis. Parathyroid hormone is released from parathyroid glands. FGF23 is released from osteocytes and 1,25-dihydroxy vitamin D is synthesized in the tubular epithelium. (Red arrows- direction of phosphate movement. Blue arrows - regulating factors)

important phosphaturic factor and decreases the reabsorption of Pi from the glomerular filtrate. Hence, with a low Pi diet more Pi is reabsorbed from the filtrate (see Figure 3). Similarly, serum Pi concentration is regulated with high Pi diet or with hyperphosphatemia resulting in increased PTH secretion and Pi excretion.[5] With all these regulations, the skeletal system maintains a role in both acute and chronic Pi balance. Thence the chronic dysregulation of Pi balance often manifests with skeletal pathology; with a decreased bone mass in hypophosphatemia presenting as rickets or osteomalacia, and in hyperphosphatemia with pathological calcifications.

Other known and unknown factors serving important roles are involved in this classical and well-studied model of Pi homeostasis. Notable among them are the phosphatonins, which were first described in reference to tumor induced osteomalacia and the resulting Pi loss through the kidneys. Since then many phosphatonins have been discovered and studied, including Fibroblast Growth Factor 23 (FGF23), secreted Frizzled Related Protein 4 (sFRP4) and Matrix Extracellular Phosphoglycoprotein (MEPE).[5] They have been described to have roles in tumor induced osteomalacia and other less common genetic diseases, and have also

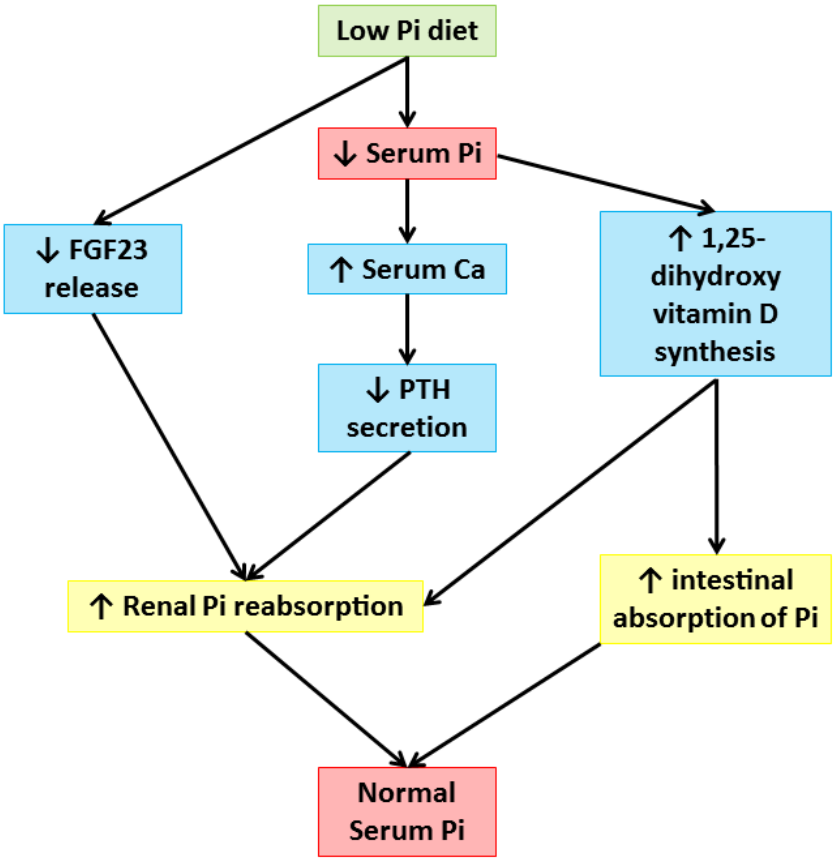


Figure 3 : Response to low Pi diet.

In response to low Pi diet, Vitamin D secretion increases and it stimulates GI Pi absorption. There is also a decreased secretion of FGF23 and PTH and a consequent increased reabsorption of Pi from tubular epithelium. (Modified from Berndt *et al*. 2007)

important roles in physiological Pi balance.[5] FGF23, for example, increases Pi excretion in the kidneys and decreases its serum concentration. It also inhibits synthesis of 1, 25-dihydroxy vitamin D by preventing hydroxylation in the kidney. 1, 25-dihydroxy vitamin D however increases FGF23 synthesis.[5] FGF23 is also regulated by PTH (in a similar way as 1, 25-dihydroxy vitamin D) and dietary Pi intake (see Figure 4).[8] FGF23 is positively correlated with serum Pi in CKD and is suggested to precede the rise of serum Pi.[8] Higher FGF23 is also correlated with the progression of CKD[3] and can play an alleged role in development of secondary hyperparathyroidism by inhibition of vitamin D synthesis. Hence, FGF23 has been suggested to be a better marker than serum Pi, which is not indicative of the body Pi load, and also possibly a better marker in the progression and as treatment target in CKD.[9] Indeed, vascular calcification is associated with normal Pi levels, but high FGF23, in cases of early CKD.[10]

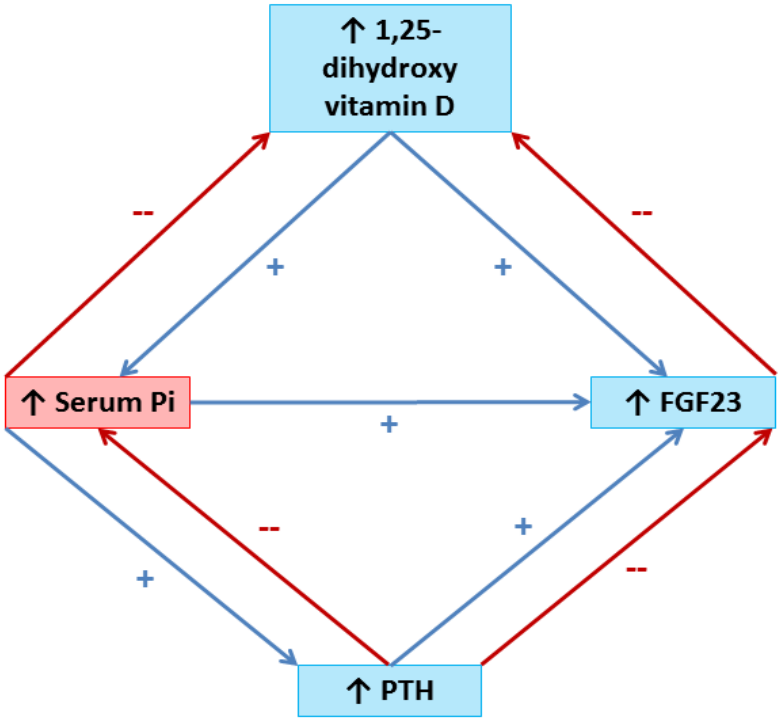


Figure 4 : Reciprocal regulation by various regulatory factors involved in Pi homeostasis.

The factors involved in Pi homeostasis are regulated by each other to maintain a tight control. PTH: Parathyroid Hormone, FGF23: Fibroblast Growth Factor 23. (Modified from Berndt *et al.* 2007)

1.3. Dysregulation of Pi homeostasis in health and in disease, and its negative impact

The dysregulation in Pi homeostasis follows a feed-forward loop, with an independent progressive failure of the renal function. This abnormality, however, is often not recognized in the early stages of CKD. A short term Pi balance study did not find any evidence for a

significant positive Pi balance in 3/4 stage CKD.[10] Recent compelling evidence suggests Pi as a direct risk for cardiovascular mortality in normal patients[11], which has also been linked to age-related vascular calcification.[12] There is ever increasing amount of Pi in the diet with increase in meat consumption and processed food. Pi additives in foods serve as buffering substance and are used frequently in high quantities. With a higher bioavailability than natural Pi (90% compared to 40-60%)[13], they increase the dietary Pi by as much as 70%.[14] This higher dietary intake of Pi is associated with high, supra-normal postprandial spikes in serum Pi, and can induce endothelial dysfunction.[15] A recent study showed significant increase in serum Pi for 10 hours after the meal in healthy women given Pi additives containing food; and this dietary spike was positively associated with cardiovascular events.[16] Similar spikes in hyperphosphatemia could be more harmful in CKD patients with an increased spike duration due to decreased Pi excretion.[7]

Targeting reabsorption in the kidneys is not very successful as treatment of hyperphosphatemia with the failing renal function. Dialysis is effective in removing only 70% of excess Pi load in patients with ESRD.[17] In various models of CKD, Pi transport across GI epithelium was maintained to normal levels; and this could implicate as an increasing load on the kidneys.[10, 18, 19] Current treatment strategies comprises of gastrointestinal inhibition of Pi absorption, indirectly with diet control and Pi binders. But, dietary Pi correlates with the nutritional value of the food, and dietary Pi restriction has been associated with a worsening malnutrition in CKD patients.[10] Older (Aluminum or Calcium containing) binders are more effective in lowering serum Pi, but are associated with toxicity. Newer binder drugs are better tolerated, but still result in considerable gastro-intestinal side effects. They are also more expensive and have frequent problems with compliance. Newest binder drugs (Sevelamer, Lanthanum carbonate) are better tolerated, but are still not free from side effects.[20] Binder drugs, in general, have the problem of frequent and high dosing, high cost, bad taste and GI side effects; all leading to a decrease in compliance. A recent study involving binder treatment and dialysis in patients of ESRD showed decreases of serum Pi to recommended levels in only 50% of cases.[21]

There is need for a newer drug, which is better tolerated, presents fewer side effects and is more effective in reducing the Pi load. In a recent study, nicotinamide showed inhibition of Pi transport in the GI tract and had beneficial effect towards the renal function, in a model of CKD in rat[6]; but has important side-effects and can be toxic. A more directed targeting of Pi absorption by blocking Pi transport through the enterocyte could be important in the treatment of hyperphosphatemia, but an incomplete understanding of gastro-intestinal Pi absorption precludes important research.

1.4. Gastrointestinal Phosphate Absorption

The GI tract plays a more important role in Pi homeostasis than previously recognized. The rate of Pi transport across the intestinal epithelium adapts efficiently depending on the dietary Pi content, showing an increase in Pi transport with low Pi diet. But, the role in CKD remains far from understood, with a normal GI Pi absorption maintained in CKD induced hyperphosphatemia. It seems to have a greater role in feed forward regulation of Pi homeostasis in bones and kidneys, depending more on luminal Pi concentration than on serum Pi. Indeed, instillation of Pi in the duodenum leads to release of a yet unknown phosphatonin like factor, which quickly inhibits Pi reabsorption in kidneys.[22] Also, dietary Pi is a much more potent stimulator of FGF23 release from osteocytes[8] than are the changes in serum Pi concentrations, which interestingly shows no significant alternations on FGF23 secretion.[23]

The small intestine is the most important intestinal segment for the GI Pi absorption, with no appreciable Pi transport seen in the stomach[24], and a small, irrelevant role of Pi absorption in the large intestine. In normophosphatemic conditions and with a regular diet, most of dietary Pi is absorbed across the intestine. But, the mechanism of Pi transport in the small intestine is not well understood. Pi transport across the enterocyte epithelium can occur through both passive concentration-dependent transport and active energy-dependent transport. The individual contribution of both these pathways is not clear, with different studies reporting different respective contributions. But indeed, the contribution found for each pathway is dependent on various factors, like luminal Pi concentration, the study model, the segment of intestine studied and experimental factors like pH and sodium (Na^+) ion content of the solution. Irrespective of the mode of entry of Pi into the enterocytes, very little is known about the Pi efflux from the baso-lateral membrane (see Figure 5).[25]

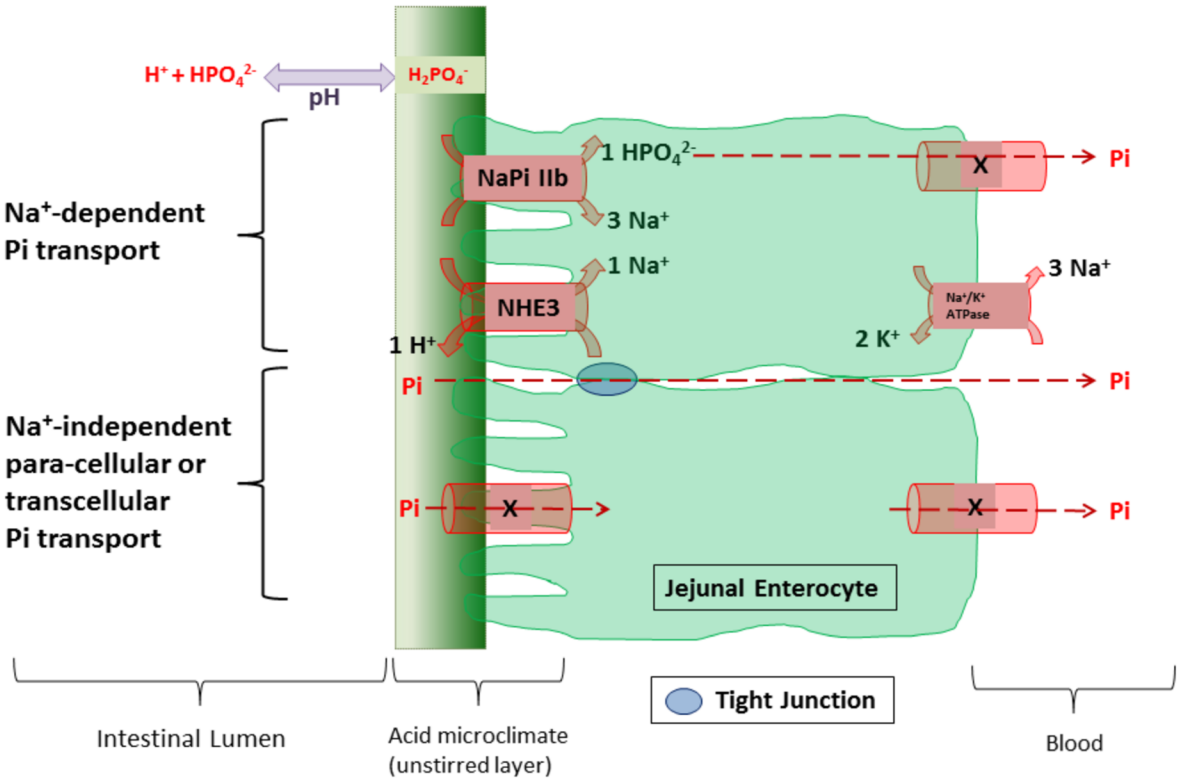


Figure 5 : Pathway of Pi absorption from the GI tract.

Pi absorption in the intestine occurs via two pathways: 1) Active sodium dependent and 2) Passive concentration dependent. Active transport is mainly mediated through NaPi IIb channel. Passive transport could be both through trans-cellular via an unknown channel or para-cellular via tight junction. (Modified from Lee *et al*. 2013)

Passive transport can be trans-cellular, through a yet unknown carrier molecule and/or para-cellular through intercellular space (see Figure 5).[10] Irrespective of the pathway, it is concentration dependent and hence a bigger contribution of passive transport is possible with a higher luminal concentration of Pi, and may not be dependent on the Na^+ content or energy (see below). The inside of the cell is negatively charged and has a Pi concentration of 0.7 – 2.5 mM; and serum Pi concentration is about 0.8 – 1.2 mM.[26] So clearly, passive transport is probably not a major contributor in low luminal Pi concentrations. Both modes of passive transport can involve un-ionized or ionized form of Pi, the concentration of which would depend on the luminal pH and osmolarity. In the presence of a negative intracellular charge and the cation selectivity of intestinal tight junctions[27] the passive transport of ionized Pi can be inefficient[28] with the transcellular- and the paracellular- passive transport respectively. However, with a high luminal Pi concentration, passive transport could contribute as a major and unregulated mode of Pi transport.[24] The normal luminal

concentration of soluble Pi is not clear and depends on the Pi content of the diet. In a study involving low to high Pi diets in patients with CKD, the jejununal luminal Pi concentration varied from 3 to 12.2 mM.[29] In a recent study in weaning rats, Pi concentration was 4.8 ± 0.7 mM with normal diet.[30]

Active transport, unlike its passive counterpart, is Na^+ and energy dependent. There are two major families of Na^+-dependent Pi co-transporters responsible for transport of Pi across the hydrophobic cell membrane, and against the electro-chemical gradient. Type-III Na^+-dependent Pi transporters, otherwise known as SLC20 transporters, include two channels: PiT-1 and PiT-2. They are expressed in most of the cell types and are responsible for house-keeping functions: like individual cellular Pi need.[31] Type-II Na^+-dependent Pi transporters (SLC34) have the role in movement of Pi across epithelial layers, and include three transporter molecules, commonly known as NaPi IIa, NaPi IIb and NaPi IIc.[26] NaPi IIa and NaPi IIc are more importantly expressed in the tubular epithelium and are responsible for reabsorption of filtered Pi load. NaPi IIb is expressed in small intestinal epithelial brush border membrane (BBM), alveolar epithelium and in testes.[26]

Like NaPi IIa, NaPi IIb is electrogenic and preferentially transports Na^+ and HPO_4^{2-} (see Figure 5) with a stoichiometry of 3:1, and with a Km value of ~30 mM and ≤50 µM respectively, at pH 7.4 when measured in *Xenopus laevis* oocyte injected with NaPi IIb cRNA.[26] Though the *in vivo* Km values are normally higher due to the presence of various barriers including unstirred layer, the above values are much smaller than the normal luminal concentration of Na^+ and HPO_4^{2-} in intestine. This means that the NaPi IIb transporters are quickly saturated and are functioning at their maximum capacity under physiological conditions, and could be responsible for only a portion of total GI Pi absorption. Indeed, in the above study for measurement of luminal Pi after low Pi diet in patients with CKD, it was as low as 0.7 mM after treatment with aluminum containing binder.[29] But, this could still be higher than Km of NaPi IIb, which would signify that the transporter is easily saturated after a normal diet. NaPi IIb activity is also pH dependent (see Figure 6), with higher activity seen with higher pH.[26] Apart, the ratio of HPO_4^{2-} to total Pi is also dependent on the pH with a higher fraction at a higher pH.[32] Both these factors combined with the specificity of NaPi IIb for HPO_4^{2-} could translate into a lower maximal activity of NaPi IIb in intact intestine, due to presence of an acid microclimate (ACM) close to the mucosa in the unstirred layer.[33] The regional expression of NaPi IIb in small intestine is species dependent with predominant expression in duodenum and jejunum in humans and rats, with almost no expression in ileum. Notably, the expression in mouse is predominantly in ileum with almost no expression in upper intestine.[34] So, in humans and rats, this expression profile of NaPi IIb combined with faster transit time of food bolus, shorter length of upper intestine and lower intra-luminal pH decreases the probable theoretical contribution of NaPi IIb mediated-active

transport in GI Pi absorption. Indeed, in studies involving bolus administration of Pi in rats showed a higher contribution of the ileum, signifying a higher contribution of passive transport in GI Pi absorption.[35, 36]

Contrary to above arguments, NaPi IIb seems to play an important role in GI Pi absorption and Pi homeostasis, based on multiple studies. In various reports Na^+-dependent Pi transport constituted 30 – 80% of total GI Pi transport[7] and NaPi IIb was responsible for up to 95% of Na^+-dependent Pi transport.[37, 38] NaPi IIb has an important role in Pi absorption in the placenta, and a constitutive knock-out (KO) is fatal in mice *in utero*.[38, 39] But, a non-developmental (inducible) NaPi IIb KO still shows normal serum Pi concentration, though with increased expression and activity of renal NaPi IIa and consequent Pi reabsorption.[37] Similar results were also seen in NaPi $IIb^{+/-}$ mice.[38] The compensatory response is possibly

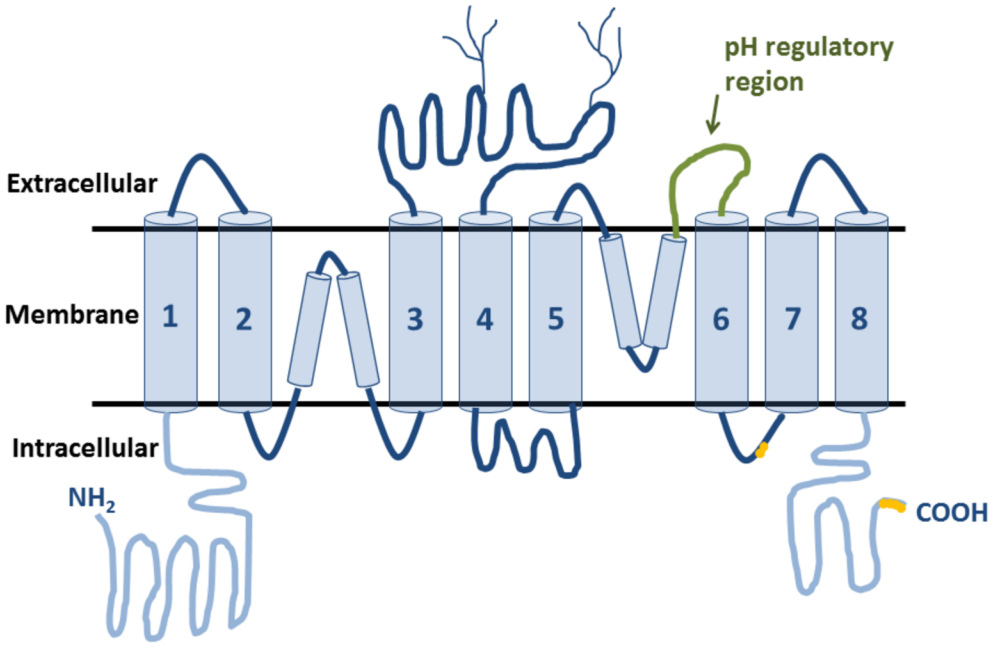


Figure 6 : Secondary structure of NaPi IIb channel.

Both N- and C-termini are located intracellularly. Green loop: pH sensitive domain. Yellow region in the C-terminal is the regulatory domain. (Modified from Wagner *et al*. 2014)

due to a decreased FGF23 seen in these mice, with an increased amount of 1,25-dihydroxy vitamin D; and based on these, NaPi IIb could be an important factor in the release of enteric phosphatonin and feed-forward regulation of renal Pi transport.[22] It would also explain the presence of normal serum Pi and Ca in people with defective NaPi IIb, who normally present with microlithiasis in lungs and testes.[40] In a recent mouse model of constitutive but intestinal specific NaPi IIb KO developed by Biber *et al*, there was increased fecal Pi excretion with decreased urinary Pi. There was also an increased expression and activity of NaPi IIa in kidneys and decreased FGF23 levels, which was significant only in

females. There was, though normal serum Pi and Ca, an almost abolished Na^+-dependent Pi transport in ileal BBM vesicles (BBMV) in both males and females.[41] However, in a model of adenine induced CKD, the mice with inducible NaPi IIb KO showed lower serum Pi and FGF23 levels than in Wild Type (WT) mice. Treatment with sevelamer had additive effect of decrease in serum Pi and FGF23, and also in bone disease, in NaPi IIb KO but not in WT mice with CKD.[42] Irrespective of the role NaPi IIb plays in GI Pi absorption, it could still be an important target in CKD; modulating the Pi homeostasis and decreasing FGF23 release. NaPi IIb itself is also regulated with the Pi content in diet; for example, there is an increased expression and activity with low Pi diet, which is mediated both with and without vitamin D.[43, 44] There is a lack of studies and a specific NaPi IIb inhibitor, which restricts our understanding of GI Pi absorption and a potential role of NaPi IIb in CKD, pathophysiologically and therapeutically. There has been a recent interest in the physiology of GI Pi absorption and also in other possible targets against CKD hyperphosphatemia, which could indirectly decrease Pi absorption.

A notable candidate for this role is Na^+/H^+ exchanger isoform 3 (NHE3). NHE3 is important in the nephrons and BBM of enterocytes, and plays role in maintenance of pH, Na^+ and water absorption, among many other functions.[45] There has been a recent interest in NHE3 as a target against other complications of CKD, like volume overload, hypertension and inter-dialytic hypotension. It is fueled by the development of a new group of NHE3 inhibitors by Ardelyx Inc., which are not absorbed from the intestinal tract and inhibit NHE3 locally; making them an intestinal specific NHE3 inhibitor.[46, 47] Notably, the drug had important beneficial effects like decrease in fluid overload, ventricular hypertrophy, blood pressure and albuminuria, in a nephrectomized rat model of CKD when administered both prophylactically or therapeutically.[46] Along with the other findings, there was also an increase in loss of Pi ion in stool, which was found serendipitously. This was interesting and could not be directly explained with NHE3 inhibition. In another study conducted with the similar drugs, in normal rats and in a $5/6^{th}$ nephrectomized model of CKD with vascular and end-organ calcification, the inhibitor drugs decreased GI Pi absorption, decreased and increased urinary and fecal Pi excretion respectively, decreased FGF23 levels, and most significantly also diminished the vascular and end-organ calcifications.[48] They also had a slowing in progression of the kidney disease which could be explained by inhibition of NHE3, but the mechanism of decrease in GI Pi absorption remained elusive. This could be important in our understanding of the physiology of GI Pi absorption and also in the treatment of hyperphosphatemia in CKD. It was also clear with these studies that NHE3 inhibitors did not inhibit the NaPi IIb transporters directly, but it was unclear, if the inhibition of Pi absorption takes place in NHE3 and NaPi IIb co-expressing intestinal epithelium. If yes, the mechanism of decrease in Pi absorption with NHE3 inhibition is

something to look into. It is important for us to understand the mechanism of this inhibition and the role, if any, that NHE3 plays in GI Pi absorption. This requires a basic understanding of NHE3 itself, its role in GI physiology and the possible ways it could affect GI Pi absorption.

1.5. Na^+/H^+ Exchanger isoform 3 and its potential role in regulation of gastrointestinal phosphate absorption

NHE3 (SLC9A3) is one of the nine mammalian isoforms of Na^+/H^+ exchanger transporters (SLC9 or NHEs). It is an epithelial molecule which is important in the GI tract and kidneys. Both in the intestine and the proximal tubule, its most important role is the electroneutral absorption of NaCl. In the intestine, this is accomplished by a functional linkage with other anion transporters, like DRA and PAT-1.[45] NHE3 can also be functionally uncoupled and has important role in absorption of D-glucose and peptides (see Figure 7).[45] It is expressed throughout the length of small intestine and also in the colon.[49] Signifying the role of NHE3 is a model of NHE3 KO mice, which presents with increased stool water, modest diarrhea, low blood pressure and mild acidosis.[50] This role of NHE3 in water and Na^+ absorption, maintenance of acid/base status and nutrient absorption could be important in the regulation/facilitation of GI Pi absorption; though a direct significance is yet to be recognized. The role of NHE3 in maintenance of pH is significant. In enterocytes, it helps in the correction of pH after nutrient absorption[51] and also has a role in the maintenance of acidic pH in the unstirred layer close to BBM.[52] Modulating HCO_3^- absorption in the tubular epithelium, it also has a role in keeping the systemic pH towards normal.[45] NHE3 itself is highly regulated by many intracellular factors known as NHE Regulatory Factor (NHERF), which include NHERF1, NHERF2, PDZK1 and IKEPP, among other regulatory factors.[53] Many of these factors also regulate other transport processes and are important mediators in regulatory interaction between transport molecules. NHE3 is also connected with the cytoskeleton[45], which is an important component in the regulation of membrane transport.

Thus, the role NHE3 plays in GI Pi absorption, and hence in its physiology and also in CKD hyperphosphatemia can be due to any of the diverse possibilities mentioned below:

- **Modifying fluid absorption and motility of the intestine:**

  NHE3 is important in intestinal NaCl and fluid absorption. Inhibition of its function leads to increase in intra-luminal volume, which consequently stimulates the intestinal motility.[50] So, inhibition of NHE3 indirectly leads to decreased transit time of food through the intestinal tract, which would also decrease the available

concentration of Pi. This could affect both active and passive component of GI Pi absorption.

- **Modification of intra-luminal pH, and the pH or composition of acid microclimate:**

  NHE3 generally transports Na^+ into the cell in exchange of transporting H^+ out of the cell. This function is important for the maintenance of pH in the micro-climate of BBM and also affects the intra-luminal pH (see Figure 5). In the above mentioned model of NHE3 KO mice, the stool was more alkaline than WT mice.[50] Also, in the studies with intestinal NHE3 inhibitor, the stool was more alkaline with the use of the drugs.[46] This change in luminal pH could affect the concentration of HPO_4^{2-} ion and also the activity of NaPi IIb channels, both of which are dependent on the pH. Again, the ionization of Pi is affected, which could influence passive Pi transport.

  The enterocyte BBM is covered with a thin layer of unstirred layer of mucus, which is more acidic than the real lumen. There are reports of a role of NHE3 in the maintenance of this layer and also in keeping the pH acidic.[33, 52] This could also affect the GI Pi transport in a similar fashion.

- **Indirect regulation of active Pi transport:**

  NHE3 is important in regulation of many other intestinal transporters. In the enterocyte BBM, NHE3 colocalizes with peptide transporter, PepT1.[54] PepT1 is involved in H^+ coupled peptide transport into the cell and consequently leads to an intracellular acidification. NHE3 is then activated and is useful in maintaining the intracellular pH towards normal. Inactivation of NHE3 leads to an indirect decrease in peptide absorption.[51] Similarly, NHE3 colocalizes with other transporters and is important in electroneutral absorption of NaCl in the intestine. There are varying reports of coupled-regulation of SGLT1 and NHE3 transporters, showing increase in NHE3 activity with activation and/or stimulation of SLGT1 transport.[55, 56] Though NHE3 generally transports Na^+ into the cell and H^+ out of the cell, the reverse is also possible (see Figure 7).[45] And, the possible role of NHE3 in regulation of NaPi IIb in any of the above manners cannot be ruled out.

- **Indirect regulation of passive Pi transport:**

NHE3 has been described to play roles in stimulation of different passive transports. Ca^{+2} reabsorption in tubular epithelium is a good example. Na^{+} transport has been described to serve as a driving force for passive Ca^{+2} transport across tubular epithelium.[57] Intestinal NHE3 is reported to be regulated by vitamin D and PTH.

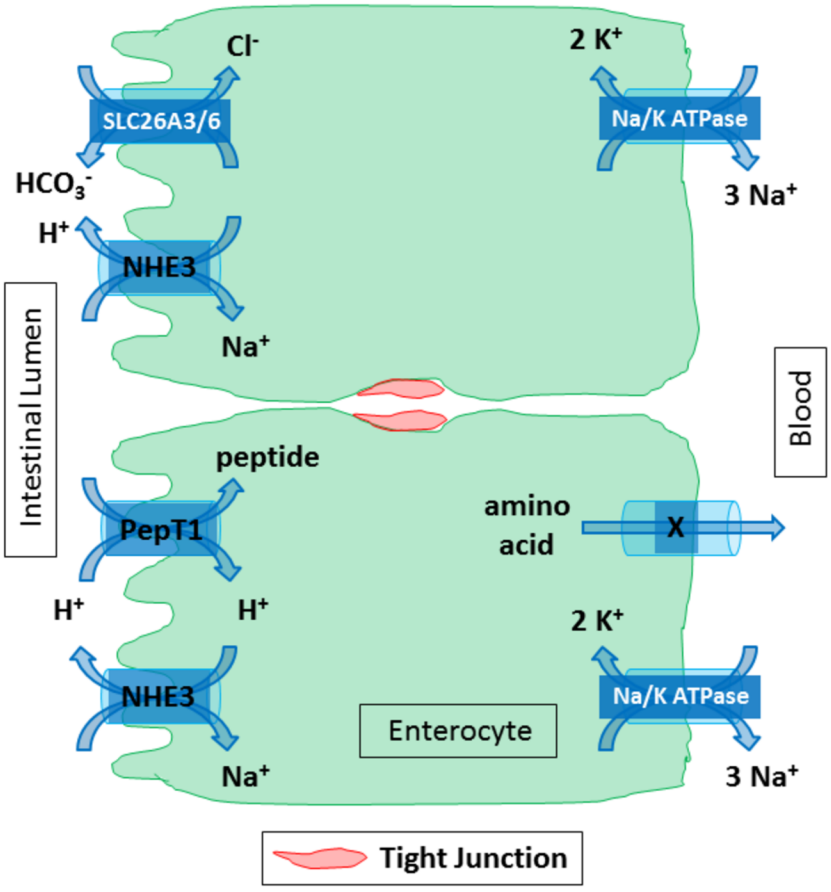


Figure 7 : Role of NHE3 in NaCl and peptide absorption.

Upper cell: NHE3 couples functionally with SLC26A3/6 channel to transport NaCl electroneutrally. SLC26A channel involved, transports chloride into and bicarbonate out of the cell (the values do not represent the actual stoichiometry). Both these channels are secondary active transporters and are dependent upon Na/K-ATPase in the basolateral membrane. Water molecules are transported passively along with charged ions.

Lower cell: NHE3 is useful in the correction of acidity with proton transported in to the cell with peptide transport, mediated by PepT1 channel. Similarly, they are secondary active and energy is spent on Na/K-ATPase.

However, the role it plays in passive transport of Ca^{+2} (in the intestine), if any, is not completely understood. NHE3 generates an osmotic gradient of 12 mOsm in the tubular epithelium, and this could drive passive movement of ions.[57] Similar

decrease in resistance to passive transport with activation of Na^+ coupled nutrient transport increases the flux of some inert solutes (which also activates NHE3; see above). This can also be achieved by the dilatation of tight junctions (TJs), reported with electron microscopy.[58]

- **Regulation through regulatory factors:**

  Various regulatory factors, like NHERFs, are useful in the regulation of NHE3 expression and activity. Many of these regulatory molecules are shared with other transporters and could be useful in an inter-regulation with NHE3.[53] One good example is CFTR, which also associates with NHE3, and in a way competes for one of these NHERFs with NHE3.[59] There can also be an activation of NHE3 with an activation of SGLT1, which is mediated via NHERF (see Figure 8).[56] NHERF1 interacts with NaPi IIa in tubular epithelium and is important in its regulation.[45, 60] NaPi IIb also has a regulatory domain in its C-terminal and can interact with NHERF1. NHERF1 has been described to be active in response to low Pi diet and the increase in NaPi IIb expression. NHERF1 KO mice, indeed, have a lower serum Pi than WT mice.[61] This role of NHE3 and NHERFs in regulation of active Pi transport is interesting.

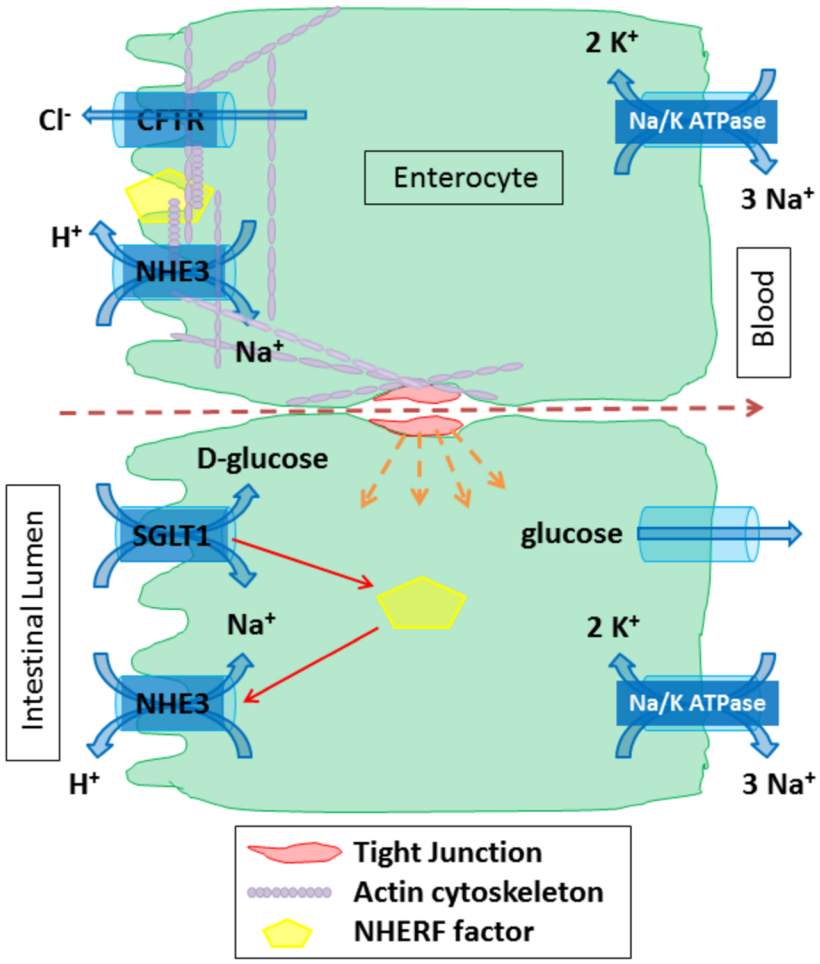


Figure 8 : Role of NHE3 in the regulation of other transport processes.

Upper cell: NHE3 associates with CFTR channel and competes for a common NHERF factor for its activity (see text). The channels in the brush border membrane are connected with the actin cytoskeleton, which maintains their stability and helps in regulation by binding to various factors. The actin cytoskeleton is also connected to tight junction molecules and is probably involved in their regulation.

Lower cell: SGLT1 activates NHERF factor in the cell which leads to activation of NHE3. SGLT1 activity and also activity of NHE3 have been described to create a paracellular solvent drag and consequent increase in paracellular transport of small molecules. (Dotted arrows represent the same, with dilatation of tight junctions; see text for references)

The regulation of TJs and their components, like claudin and occludin, is not completely understood. But, there are described changes in TJ ion-selectivity with changes in composition of these molecules. They are also connected to actin-cytoskeleton in similar way as NHE3 and its regulatory factors (see Figure 8).[58, 62] And, this could also be related to the regulation of passive Pi transport.

- **Role of NHE3 in maintenance of blood pH and effect on Pi absorption by its dysregulation:**

NHE3 is active in the maintenance of blood pH, with its physiological role in tubular epithelium, where it helps with bicarbonate reabsorption. Intestinal NHE3, though, is more significant in neutral NaCl absorption.[45] But, it is also necessary for bicarbonate absorption from the intestinal tract. Failure to do so, as seen with NHE3 KO and with intestinal NHE3 inhibition, results in intra-luminal alkalization and mild acidosis.[46, 50] There is also a small drop in serum bicarbonate. Though small, this could affect intestinal Pi absorption. Metabolic acidosis, for example, stimulates intestinal NaPi IIb.[63] Pi is an important titratable acid and is important buffer in the body. This role of NHE3 can have implications in Pi homeostasis and absorption.

It is clear that NHE3 could be an important regulator of GI Pi absorption. A direct role, however, is yet to be recognized. But, it is clear that NHE3 is a key facet in its understanding. An inhibitor of NHE3 could also serve as a useful target for CKD hyperphosphatemia. With incidental findings and also with elaborate studies[46, 48], this definitely seemed to be the case. But, it is important for us not to overanalyze such results, as these studies could often be misleading (see Discussion). Results of short-term study-models and effects might not translate into similar effects in a gradually developing CKD in humans. More research towards the same is vital.

2. Aim of the Study

The primary aim of this study includes:

- Understanding the role of NHE3 in intestinal phosphate absorption, and
- Validation of NHE3 inhibitor as a potential drug target against hyperphosphatemia in CKD.

  To answer our primary questions, we focused on to the following:

  - Effect of inhibition of intestinal NHE3 transporter on phosphate absorption and fluid absorption in an intestinal epithelium expressing both NHE3 and NaPi IIb transporters, and
  - Studying the role of pH, with changes in pH of intestinal luminal milieu and/or due to intestinal NHE3 inhibition, on GI Pi absorption.

3. Materials and Methods

3.1. Chemicals and Drugs

A non-absorbable inhibitor of NHE3 transporter was used for the experiments in this study, which was kindly provided by AstraZeneca, Sweden. Due to the experimental nature of this drug, it is called AstraNIhn (AstraZeneca NHE3 Inhibitor) in this report, for clarity. AstraNIhn was used as per manufacturer's instructions and belongs to a group of drugs developed by Ardelyx Inc. and the studies conducted therewith.[46-48] Two forms of AstraNIhn were used during the course of the study, i.e. AstraNIhn salt and AstraNIhn base (described in results section).

Bovine albumin (A3912), atropine (A0132), dimethyl sulfoxide (D4540), Gly-Sar (G3127) and the anesthetic 5-ethyl-5-(19-methyl-propyl)-2-thiobarbiturate (Inactin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heparin and metamizol were purchased from Ratiopharm GmbH, Germany. S1611 was a kind gift from Sanofi-Aventis GmbH, Germany. S1611 is a selective inhibitor of NHE3 transporter, which is absorbed systemically and hence inhibits NHE3 in both intestine and kidney.[50] ^{3}H labeled Polyethylene Glycol (^{3}H-PEG 4000) and ^{32}P-Phosphoric acid were purchased from Hartmann Analytic GmbH, Germany. All other chemicals were obtained from Applichem GmbH, Germany, unless mentioned otherwise.

3.2. Ethics statement

All studies were approved by the local institutional animal care and research advisory committee at the hannover medical school and authorized by the local government for the regulation of animal welfare (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES). At the conclusion of the experiments, rats were sacrificed with CO_2 narcosis followed by cervical dislocation. The experimental procedures performed and types of anesthesia used were based according to university and national guidelines and are explained below.

3.3. Study model

The rat model of *in vivo* intestinal perfusion was used for most of the experiments performed in this study. Rat, as an experimental animal, was selected as they are close to humans in their expression profile of NaPi IIb and GI Pi absorption[34], and the ease of handling. *In vivo* intestinal perfusion model was adapted from the group of Sjöblom *et al*.[64]

and was selected as it preserves the neuro-humoral regulation, which is particularly important in Pi absorption and homeostasis. For the experiments involving Pi absorption, a segment of jejunum was utilized, as it is the segment involved in most of the active Pi absorption. Few experiments were also performed in distal ileum to study the inhibition of NHE3 on fluid absorption in that segment.

A few initial *ex vivo* Ussing Chamber experiments were also performed in the mouse ileum, as it is the segment of intestine responsible for active Pi transport in mice.[34] Another set of experiment was performed in the jejunum. But this model was not used for further experiments due to its complexity with the use of radioactivity and also the brevity of electrical response elicited with physiological concentrations of Pi. As these experiments constitute a small portion in this report, their procedure is described only briefly, for reader's understanding.

3.4. Animals

Male Sprague-Dawley rats weighing 300-400 g were obtained from Janvier labs, Saint-Berthevin Cedex, France. For few experiments, male Sprague-Dawley rats were obtained from the animal facility at MHH (Zentrales Tierlabor; ZTL). The rats were kept in groups in cages and had free access to water and standard rat chow *ad libitum*. They were maintained in standardized conditions (12:12 light:dark cycle, 20-21°C). The animals were acclimatized to the new environment for at least 5 days, before experiment. The age of rats during experiment is mentioned in the results under each experiment. Prior to surgery, they were fasted for 16h, with free access to drinking water.

For Ussing chamber experiments, C57BL/6 mice were obtained from ZTL and were treated in a similar manner as the experimental rats. But, they were fasted for 10h before sacrifice.

3.5. Surgical procedure

The rats were anesthetized with 125 mg/kg inactin given i.p. after an induction with 1.5% inhaled isoflurane. The body temperature was maintained at 37-38°C with a rectal thermistor-controlled warm mat and an infra-red lamp, during surgery and throughout the experiment.

All experiments were started between 9 AM to 11 AM, to prevent any effect of daily circadian changes. The animals were tracheostomized and a small polyethylene tube (PE-320; Becton, Dickinson & Co., Franklin Lakes, NJ, USA) was inserted in the trachea to facilitate respiration. The left and right femoral arteries and the right femoral vein were catheterized with PE-50 polyethylene tube (Becton, Dickinson & Co., Franklin Lakes, NJ,

USA). The arterial catheters were filled with isotonic saline containing 20 IU/mL heparin, and one of the catheters was connected with a Blood-Pressure transducer operating with PowerLab system (AD Instruments, Hastings, UK), for a continuous monitoring of blood pressure. A mid-ventral celiotomy was performed and the common bile duct was catheterized with PE-10 polyethylene tubing (Becton, Dickinson & Co., Franklin Lakes, NJ, USA), near its entrance to the duodenum, to prevent pacreatico-biliary juice from entering the duodenum. A segment of the jejunum (starting 5 cm from the duodeno-jejunal ligament) was cannulated with Silastic tubing proximally (I.D. = 0.09 cm) and distally (I.D. = 0.3 cm) and secured with sutures. The bowel was ligated proximal and distal to the test segment to avoid any spillage. The segment was flushed with warm (37°C) saline. The proximal duodenal cannula was connected to a peristaltic pump (Gilson minipulse, Villiers, Le Bel, France) and the segment was continuously perfused with test solution at a constant rate, depending upon the experiment. The solutions were kept warm at 37°C in a water bath and a short segment of the proximal tubing was looped inside the abdomen, to make sure that the experiments were performed at a physiologic temperature. To conclude the surgery, the intestinal segment was carefully placed inside the abdominal cavity and the abdomen was closed with continuous sutures. After the surgery, the animals were rested for ~60 min for cardio-vascular, respiratory and intestinal recovery, before beginning the experiment.

Similar procedure was used for the experiments involving ileum. A 10 cm length of distal ileum, measured from close to the cecum was cannulated. For these experiments, the bile duct was not cannulated.

For experiments with collection of urine, urinary bladder was cannulated with PE-80 polyethylene tube (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). Metamizol 200 mg/kg was given with single s.c. injection to alleviate pain during surgery. Atropine 0.05 mg/kg was given with single s.c. injection, prior to Inactin anesthesia to prevent intestinal contraction and secretion caused by anesthetic medications, in few experiments. (Important details are described under each experiment.)

3.6. Experimental protocol & solutions

The necessary modifications of the experimental protocol are described separately under each experiment. The isolated loop of rat jejunum was perfused with isotonic, 2 mM phosphate containing solutions and studied for fluid and phosphate absorption per length of the intestine. Radioactive phosphate (^{32}P) and/or radioactive tritiated PEG (^{3}H-PEG) was added in the perfusion solution for the measurement of phosphate absorption and fluid flux respectively, in most experiments. For all experiments, arterial blood pressure (mmHg) and

temperature (°C) were measured continuously. S1611 and AstraNIhn were dissolved in DMSO and the final concentration of DMSO in test solution was kept at 0.05%. The solutions for control experiments or for baseline absorption contained 0.05% DMSO.

Most perfusion solutions for *in vivo* experiments were buffered with either bicarbonate/CO_2 or HEPES buffer and contained 2 mM phosphate. The phosphate was a mixture of Na_2HPO_4 and NaH_2PO_4 salts, the ratio of which was calculated based on the target pH of the solution at 37°C. The osmolarity of the solutions were always kept at 310 mOsm by adjusting the concentration of NaCl and/or mannitol; and the concentration of NaCl was always kept above 76 mM, in consideration of the Km of NaPi IIb.[26] To compensate for fluid loss during surgery and the experiment, isotonic, bicarbonate buffered fluid was infused via the venous catheter at a rate of 1 – 2 mL/300 g body weight/h. The concentration of bicarbonate and the speed were adjusted during the course of the study based on the size of the animal, blood pressure, hematocrit and blood-gas analysis (details of which can be found in the results section). The venous infusion solution also contained 2.25 mM KH_2PO_4 and 3 mM KCl, and the osmolarity was adjusted with NaCl. Whenever a blood sample was taken, equal volume of 7% bovine albumin was infused via the same catheter.

Blood-gas analysis was done with ABL5 Blood gas analyzer (Radiometer Copenhagen, Denmark) according to manufacturer's instructions. Hematocrit was measured by spinning a blood sample in a pre-heparinized Clinitube (Radiometer Copenhagen, Denmark) at a speed of 13,000 rpm for 5 minutes, in Hematocrit 210 centrifuge (Hettich Zentrifugen, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany).

The phosphate solution for Ussing chamber experiments was prepared similarly (as above). Modified Ringer's solution was used for the basal period of experiment and for the preparation of tissue, except for KH_2PO_4, which was excluded. Details of these solutions can be found in a previous publication from our group.[27]

3.7. Measurement of phosphate absorption

Phosphate absorption was measured from the loss of radioactive phosphate (^{32}P) during perfusion. Phosphate absorption was considered as the difference between the total Pi in the influent (i.e. Pi concentration multiplied with total volume of influent) and the total Pi in the effluent, divided with the volume of fluid absorbed in the same segment. Hence, the calculated volume of fluid flux was utilized for calculation of the phosphate absorption. All results of phosphate absorption were expressed as amount of phosphate absorbed per cm of intestine per hour (nM/cm/h). Results of phosphate absorption were also evaluated as the total percentage (%) of phosphate absorbed from the intestine.

3.8. Measurement of fluid flux

Fluid flux across the jejunum was calculated as the difference between the volume of influent and the volume of effluent. Volume of effluent was measured directly by gravimetric method, based on the volume of fluid collected, and indirectly by tritiated tracer method (depending on the experiment, details described below). For the volumetric method, volume of the effluent was calculated as the difference in weight of the tube with and without the collected effluent. All volume measurements were corrected for density, with the density of normal saline set arbitrarily to 1.0. For tritiated tracer method, the volume of the effluent was calculated based on the concentration of the tracer in the influent and the effluent, and considering no absorption or loss of tracer during perfusion. Effluent was collected every 10 to 20 minutes in a plastic tube, depending on the experiment. The volume of influent was calculated as the average volume from the peristaltic pump, of two samples taken immediately before and after the perfusion experiment, in both methods. All results of fluid absorption were expressed as fluid volume per cm of intestine per hour (µL/cm/h).

3.9. Measurement of intestinal contractions

For the measurement of intestinal contraction, a similar procedure was used as described by the group of Sjöblom *et al*.[64] Intestinal contraction was assessed with the changes in intra-luminal hydrostatic pressure. The proximal perfusion tube was connected to a T-tube (near its entrance into the test segment), which was connected to a pressure transducer (similar to the blood pressure transducer described above and measured similarly). The tubing and the transducer were adjusted to remain at same height as the inlet and the outlet tubes. The measurements were recorded (as the blood pressure) on LabChart 8 software (AD Instruments, Hastings, East Sussex, UK). An upward deflection of at least 2 mmHg above the baseline (over 10 minutes) was considered as a contraction.

3.10. Brief description of Ussing Chamber experiments

For detailed description of Ussing Chamber experimental protocol, the reader is referred to previous publication from our group.[27] The mice were killed by cervical dislocation after a brief CO_2 narcosis. The abdomen was opened, and the intestinal segment was resected and was immediately kept in ice cold, oxygenized modified Ringer's solution. The segment was opened along its mesenteric attachment and stripped of the serosal layer. The mucosa was

then mounted in an Ussing chamber and bathed with a bicarbonate containing solution, continuously gassed with 5% CO_2/95% O_2 on both sides. For each mouse, at least 3 chambers were used. The studies were conducted in a voltage-clamped condition, and after stability of electrical parameters and 30 min of additional baseline measurement, a test amount of Pi was added in the luminal side (see the results section), with mannitol added to the basolateral side for osmotic balance. The short-circuit current (Isc) response was measured during baseline and after addition of Pi to the solution, to see an effect of Pi transport.

3.11. Collection and Analysis of Data

For *in vivo* perfusion experiments: The volume of the effluent was measured every 10 to 20 min and 0.2/0.1 mL of the sample was mixed with 10 mL of counting cocktail (UltimaGold, Perkin Elmer, Shelton, CT). Paired samples were taken for each interval and counted with Perkin Elmer Tricarb 2800TR liquid scintillation counter (Perkin Elmer, Shelton, CT). The mean of both samples was taken as final disintegration per minute. One or more paired samples of influent solution were also taken for counting the influent solution. A 10-20 min period was allowed for the establishment of a steady state at the beginning of the experiment and also after addition of the drug (details below). For the calculation of phosphate absorption, the average of fluid flux in that period was utilized. The values of fluid flux and phosphate absorption at different time periods were averaged in each animal (for baseline only, baseline and drug-affected and drug-affected only absorption; depending on the experiment). Finally, the overall mean absorption/hour was calculated for each rat.

3.12. Statistical Analysis

All statistical analyses were performed using paired Student's t-tests to compare parametric data between groups. A P value of <0.05 was considered significant. All data are reported as the mean ± standard error of the mean, and the number of rats mentioned as 'n' number.

The experimental protocols were changed slightly during the course of the study, based on the results and the challenges. Keeping the complexity of same in mind, a brief experimental protocol (changes) and a brief discussion precedes and follows some results (in the following section) respectively, whenever deemed necessary. In the absence of a sufficient experimental number, to be able to perform a reliable statistical analysis, no such analysis was performed and is mentioned under each legend.

4. Results

4.1. Preliminary and Validation experiments

Preliminary Ussing chamber experiments were done with mouse jejunum and ileum. It showed a very small response with addition of physiological concentration of inorganic Pi. Hence, this model was not used for further studies. The results are not shown.

In vivo perfusion model in rat was selected for further experiments for its ease, cost-effectiveness and improvements in experimental parameters.

4.1.1. Validation of in vivo perfusion model in rat

It was necessary for us to validate the model for fluid and Pi absorption and for the experimental stability of rat. This is also important in our study, as changes in vital parameters could independently affect Pi absorption and homeostasis. So, pilot experiments were performed in 8 weeks old Sprague-Dawley rats. Approximately 20 cm length of the jejunum was isolated and was perfused with test solution at a speed of ~13 mL/h for a total duration of 60 minutes. The solution was bicarbonate buffered to pH 7.4 and contained ^{32}P (up to 200 µCi/100 mL) and 0.05% DMSO. Normal saline, at pH 7.4, was infused via the venous catheter for the correction of fluid loss, at a rate of 1 mL/300 g body weight/h. The effluent was collected every 10 minutes, and also blood and urine samples were collected every 10 and 30 minutes respectively (with one blank sample collected before the commencement of perfusion experiment). All the samples were analyzed for absorption of Pi. Blood sample was also analyzed for stability of the rat, with blood-gas analysis and hematocrit. The fluid flux was calculated by gravimetric method.

For absorption of Pi, experiments were done with or without 20 µM S1611 (a systemic NHE3 inhibitor; n = 3 + 3). In the S1611 group, the drug was added to the perfusion solution before commencement of the experiment. As part of a preliminary experiment, the number of experimental animals was kept low and no statistical significance analysis was performed.

In both control group and S1611 treated group, the fluid and Pi absorption were stable over 40 min period, with not much variation. The baseline value of fluid and Pi absorption were 78.3 ± 7.5 µL/cm/h and 448.1 ± 45.8 nM/cm/h respectively. There was a small decrease in

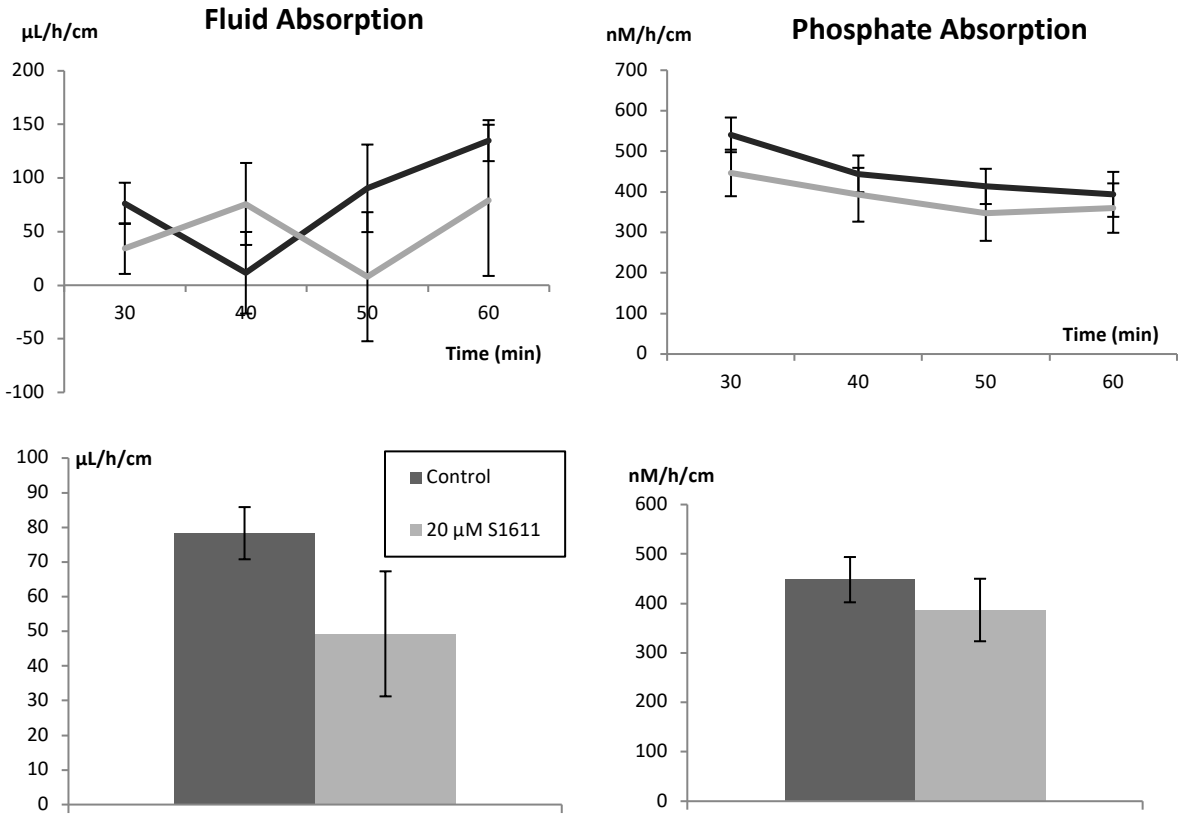


Figure 9 : Baseline Fluid and Pi absorption from the jejunum and effect of S1611.

Experiments were done in jejunum for 60 min duration and the first 20 min was considered as equilibration period. Fluid absorption was calculated by gravimetric method and Pi absorption studied with radioactive P. Upper two graphs represent variation of fluid and Pi absorption over time and lower two bar diagrams represent average fluid and Pi absorption over the 40 min experiment. The left two graphs are for fluid absorption and the right graphs are for Pi absorption. There was no significant variation of fluid or Pi absorption over time. Both fluid and Pi absorption were reduced slightly with 20 µM S1611. (No statistical analysis was performed; n = 3)

both parameters with the use of 20 µM S1611 (see Figure 9). Absorption of ^{32}P in the serum samples and excretion in urine were calculated as a percentage to the concentration in control perfusion solutions. Similar amount of ^{32}P was seen in blood (with or without S1611), but there was a higher excretion in urine of the control animals. In control animals, the ratio of ^{32}P seen in blood and urine after 60 min of jejunal perfusion was 0.21 ± 0.09 % and 48.1 ± 17.6 % respectively, of the concentration in the influent controls. This clearly shows that the absorbed Pi was rapidly excreted from kidneys (see Figure 10). With these results, it was clear that the *in vivo* perfusion model in rat is a good model for our study. But, the detection of absorbed Pi in blood was not a good parameter, due to high dilution

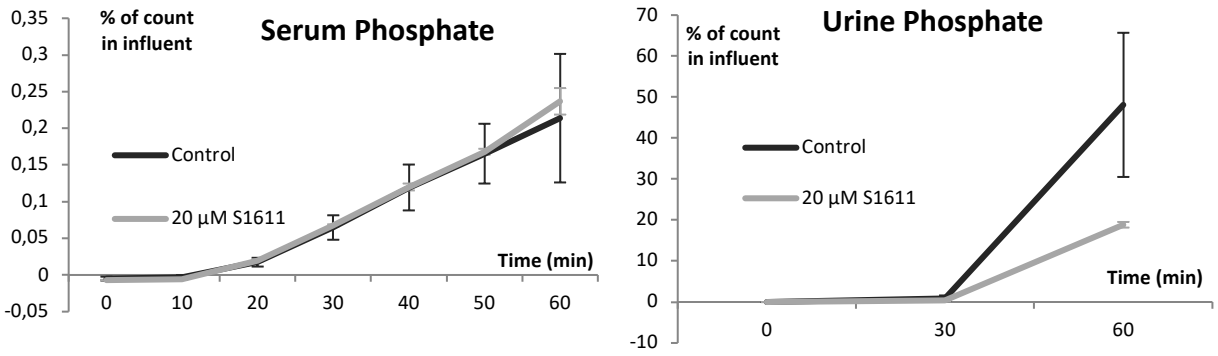


Figure 10 : Absorption of ^{32}P over time in the bloodstream and excretion through the urine.

Absorption and excretion of ^{32}P was measured over 60 min of jejunal perfusion in blood (left) and urine (right) respectively. Values were calculated as a ratio to the ^{32}P counts in the influent controls. There was not much difference in amount of ^{32}P seen in blood between the control and S1611 group; unlike the excretion in urine, which had a more rapid excretion in control animals. From both figures, it is clear that the Pi was rapidly cleared in urine after absorption from GI tract. (No statistical analysis was performed; n = 3)

and rapid excretion. As a part of the validation experiments, the number of experimental animals was limited to ~3.

Apart, blood pressure, hematocrit and blood-gas measurements were measured in control animals (n = 4). The blood pressure was measured continuously and was stable and within the normal physiologic range (80 – 120 mmHg) during the course of experiment. Blood-gas and hematocrit were measured after placement of an arterial catheter and also at conclusion of the experiment. All these values (except hematocrit) are presented in Figure 11, with an average of blood pressure values over 5 min at the beginning and at the end of experiment. The rats maintained spontaneous respiration under inactin anesthesia. The pH was maintained in normal physiologic range (7.35 – 7.45) with a slightly low sO_2 and pO_2, which improved over time. Though serum bicarbonate and pCO_2 were high in the beginning, they fell significantly; and the final bicarbonate concentration was in the low normal range (22 – 26 mEq/L). Also, there was slight hemo-concentration at the end of experimental time with slight but insignificant fall in blood pressure, which was always maintained within normal physiological range. The results of the blood-gas analysis show a compensated respiratory acidosis in the beginning (possibly caused by airway blockage, which was cleared with the tracheal tube placement), and a compensated metabolic acidosis later in the course of the experiment (possibly due to loss of bicarbonate in bile secretion). The hemoconcentration was probably a result of the fluid loss during surgery and the course of experiment.

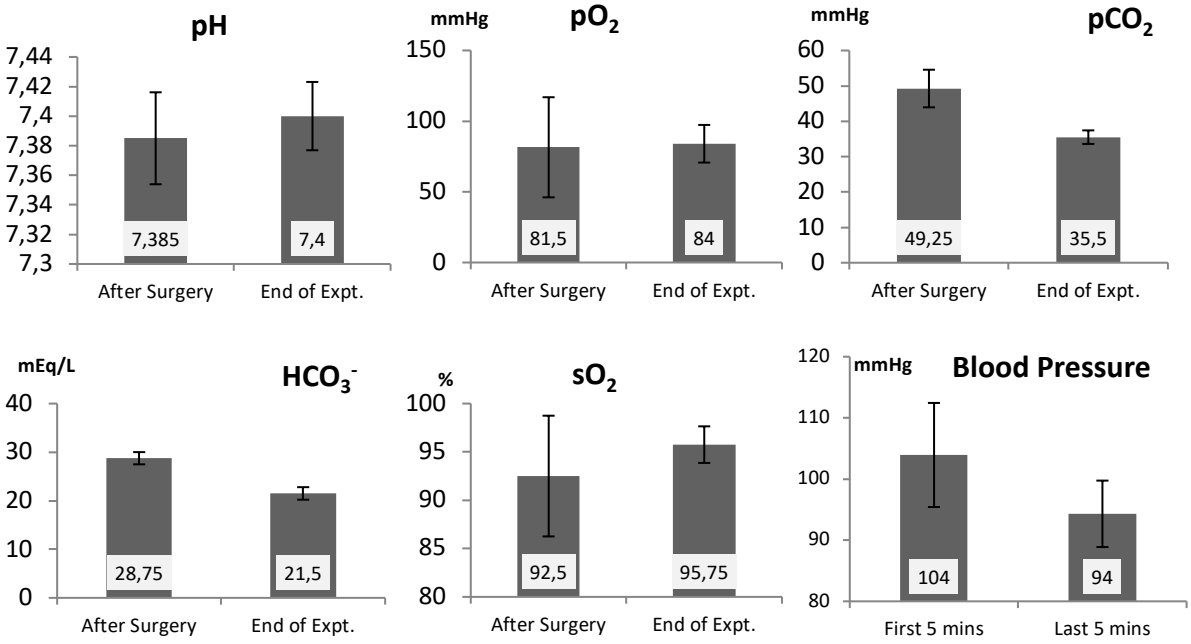


Figure 11 : Blood-gas analysis and Blood pressure measurement.

Blood-gas analysis was performed after placement of an arterial catheter and at the end of experiment. There was a compensated respiratory acidosis during surgery, with low pO_2 and sO_2, and high pCO_2 and high normal serum bicarbonate. It later converted into a metabolic acidosis, with low pCO_2 and low normal bicarbonate. sO_2 and pO_2 improved with time. The blood pressure was maintained within normal range during the experiment.
No statistical analysis was performed. (n = 4)

To counter these measures, supplemental O_2 was given through a T-tube, designed to fit the tracheal tube and bicarbonate buffered saline was infused through the venous catheter at a higher rate (1.5 mL/300 g body weight/h). In a trial with 3 rats, there was no sequential hemo-concentration with a better maintenance of blood pressure. There was also a better maintenance of serum bicarbonate with no acidosis (see Figure 12). But, there was no benefit of supplemental O_2 with only a slight improvement in sO_2, but also an increase in pCO_2. Hence, no supplemental O_2 was given in further studies (to stimulate the respiratory drive); but a bicarbonate containing infusate was continued at this higher speed.

All these results confirmed the stability of this model for our study, which was selected for rest of the study. Blood-gas analysis and blood pressure data was measured in all of the experiments, and the data is shown only in the presence of some significance. In any rare case of dysregulation of vital parameters, the rat was excluded from study. Hematocrit was not measured to decrease blood loss.

4.1.2. Validation of tracer method for fluid flux measurement

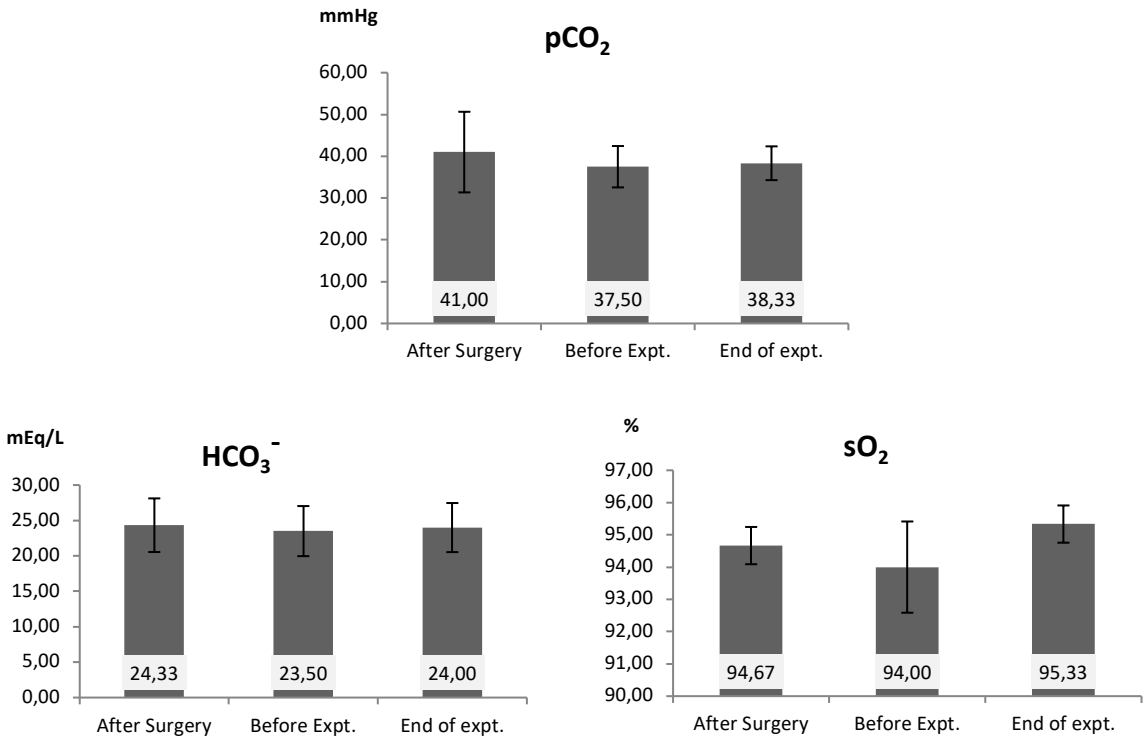


Figure 12 : Blood-gas analysis after supplemental O_2 and infusion of bicarbonate at a higher speed.

Blood-gas analysis was performed after placement of an arterial catheter, before the beginning and at the end of experiment. There is improvement in sO_2 and serum bicarbonate, with a slight increase in pCO_2 level. (No statistical analysis was performed; n = 3)

Classically, measurement of fluid flux with the use of a non-absorbable tracer in the perfusion solution is considered to be more accurate than a simple gravimetric measurement of fluid output, and is also more popularly used.[65] For the same reason, we wanted to validate our model with a non-absorbable tritiated tracer (^{3}H-PEG 4000), for calculation of fluid absorption. Experiments were done in 5 rats with bicarbonate buffered perfusion solution containing ^{32}P (up to 50 µCi/100 mL), ^{3}H-PEG (up to 10 µCi/100 mL) and 0.05% DMSO at a rate of ~18 mL/h. The study was performed in a segment of jejunum for a total duration of 60 min with a 10 min initial equilibration period. Effluent samples were collected every 10 min for measurement of intestinal fluid and Pi absorption.

As it can be seen in Figure 13, the calculated fluid flux showed a total secretion, which was stable over time. Normally, jejunum is a segment with total positive fluid absorption and this result could only be explained with an experimental error. Also, we previously found a positive fluid absorption with gravimetric method (see Figure 9). As the duodenum was cut-off from the test segment, there was no possibility of mixing of any bile juice into the perfusion solution. As our tracer has a high molecular weight of 4000 Daltons, its uptake

would not explain such big differences. The other possibility could be due to errors in counting and/or quenching.

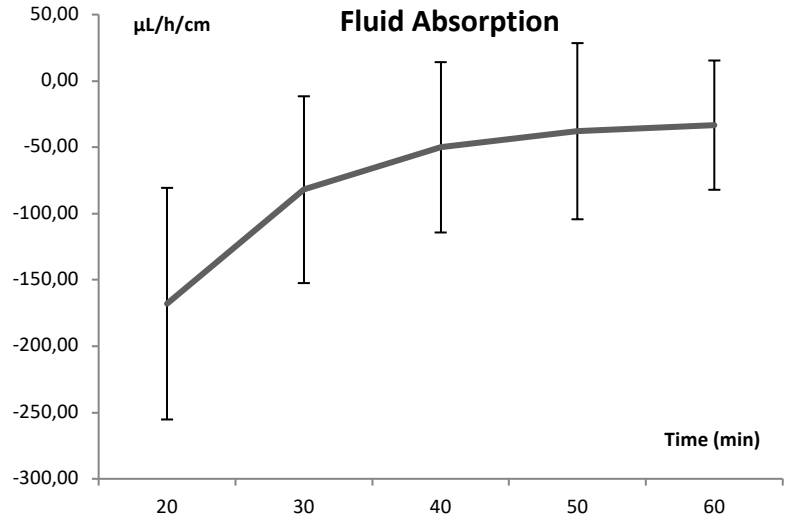


Figure 13 : Fluid flux measurement done with non-absorbable tracer method.

Fluid flux was calculated with the change in concentration of a non-absorbable tracer (^{3}H-PEG 4000) in the perfusion solution. The result shows secretion of fluid into the lumen. (n = 5)

In a series of 'Quenching' experiments, intestinal effluent did not have much effect on the measured counts with scintillation counter. But, the spectrum of ^{32}P detection range normally overlaps slightly with the spectrum of ^{3}H range. So much so that, up to 1.6% of ^{32}P counts were spilled onto the counting window for ^{3}H, in our experiment (when 200 µL samples from a 50 µCi ^{32}P/100 mL solution was measured). But, there was also up to 0.8% spill of ^{3}H counts into ^{32}P window (data not shown). With a normal fall in the counts of ^{32}P of up to 10% in the effluent (due to absorption), compared to the influent, the counts of ^{3}H measured in effluent were falsely low. This could lead to a spurious result of fluid secretion, instead of absorption. We required a high activity of ^{32}P to be able to detect the low level of phosphate absorption in blood. This problem could be solved with a gravimetric method for fluid absorption or an equal and lower amount of radioactivity; but the latter would not allow us to measure a direct detection of Pi absorption in blood. Afterwards, we tried to bypass this problem with gravimetric fluid flux measurements.

4.1.3. Effect of 10 µM AstraNIhn on Pi and fluid absorption

Experiments were performed in 8 weeks old rats with ^{32}P (up to 50 µCi/100 mL) containing solution, without any tracer for fluid flux measurement. The solution was bicarbonate buffered to pH 7.4. A small segment of jejunum (6-11 cm) was perfused at a rate of ~18 mL/h for a total duration of 90 min. An effluent sample was weighed every 10 min to calculate fluid flux with gravimetric method, with the first 10 min considered as equilibration period. Rats were randomized into two groups (of 5 animals each) and studied

for control (with 0.05% DMSO) and for the effect of AstraNIhn salt (10 µM dissolved in 0.05% DMSO).

Fluid absorption was not stable over time, especially so with the treatment group. There was no significant effect of the drug on either fluid or Pi absorption, when tested with paired Student's t-test (see Figure 14). Also, there was no obvious difference in blood or urine Pi counts with AstraNIhn (data not shown).

This apparent absence of effect of 10 µM AstraNIhn would only conclude as an insignificant inhibition of NHE3 transporters. This concentration is though much higher than the recommended concentration of the drug (1 µM), for inhibition of fluid absorption in *in vivo* situation. This absence of any effect could also result from a low penetration of the drug through mucus layer in intestine. To exclude this, we tried 50 µM concentration of the drug at a pH of 7.4. This attempt was however met with another problem. At this concentration of the drug, there were visible precipitates in solution. And again, careful observation of the 10 µM concentration solution also showed some clouding with precipitates; which was not apparent previously with naked eyes. This solubility issue of the AstraNIhn salt was not properly conveyed and was unknown to us. This problem though was not present at a lower pH. In our examination, it was completely soluble at pH of 6.8.

Apart, in the above perfusion experiment, the fluid absorption was unstable over time; especially so with the use of AstraNIhn. This could be due to any of the following factors: intestinal contractions, temporary obstructions due to either tube blockade or external pressure, sloughing off of mucus or experimental error. But, this instability also translates into the measurement of Pi absorption, as fluid absorption is factored in its calculation. To mitigate this problem, we used the average of fluid absorption over the total experimental duration (excluding the equilibration time), instead of the average fluid absorption over that particular time interval, for the calculation of Pi absorption (as also shown in Figure 14). The average of Pi absorption over the experimental duration was always very similar with both methods, though with a slight difference (and statistically insignificant, when tested with paired Student's t-test) in area under the curve (data not shown). Hence, this method was used for the rest of study. This, however, would be a lesser problem with the tracer method for fluid absorption, as the concentration of the tracer is less susceptible to such variations.

4.1.4. Effect of 40 µM S1611 on Pi and fluid absorption

As there was some insignificant inhibition of fluid and Pi absorption with 20 µM S1611,

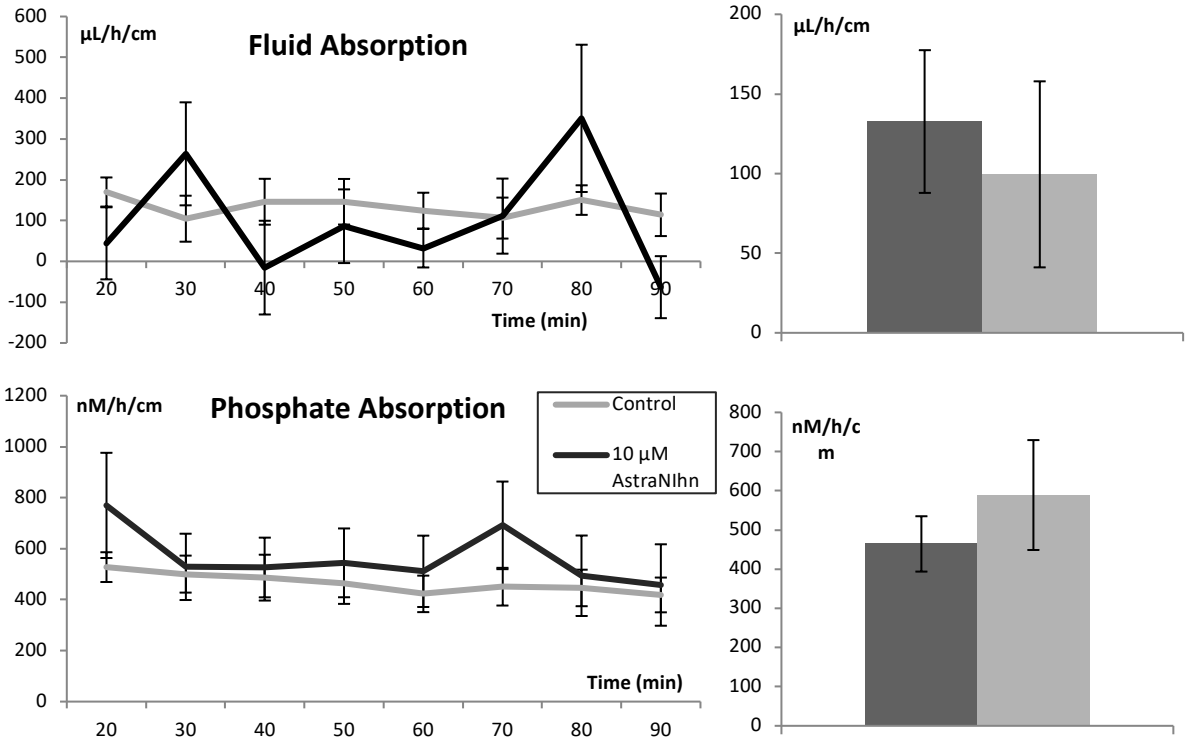


Figure 14 : Baseline Fluid and Pi absorption from the jejunum and effect of 10 µM AstraNIhn salt.

Experiments were done in jejunum for 90 min duration and first 10 min was considered as equilibration period. Fluid absorption was calculated by gravimetric method and Pi absorption studied with radioactive Pi. Upper two graphs represent variation of fluid absorption and lower two graphs represent Pi absorption. The right two bar graphs are average of absorption over 80 min. Both fluid and Pi absorption were not affected significantly with 10 µM AstraNIhn salt. Fluid absorption was not stable over time, especially, when treated with the drug. (Statistical analysis done with paired Student's t-tests; * when $P < 0.05$, ** when $P < 0.01$; n = 5)

experiment was repeated with doubled concentration. We used bicarbonate buffered solution at pH 7.4, which contained both ^{32}P and ^{3}H-PEG (up to 2.5 µCi/100 mL) and DMSO. Experiments were done in 3 rats with a short segment of jejunum (4-8 cm), which was perfused for duration of 180 min at a rate of ~18 mL/h. The drug was added to the perfusion solution at 90 min to study the effect on fluid and Pi absorption. A 15 min equilibration period was considered at the beginning and also after addition of drug in this experiment.

There was similar problem with fluid flux, with respect to a low absorption to secretion (data now shown). There were also no significant changes in the average of fluid flux, with the drug, when tested with paired Student's t-test. This similarly translated into a low Pi absorption, as seen in Figure 15. The Pi absorption however increased unexpectedly with the drug, which was not significant. But, this could not be explained based on the inhibition of NHE3 in intestine. S1611, however, also inhibits NHE3 in the kidney, which could possibly affect the intestinal Pi absorption. This drug was thence not pursued during this study. In the absence of a significant result, the experimental number of rats was limited to 3; and with a small experimental number, no statistical analysis was performed.

In a separate set of study, shorter length (4 cm) of jejunum was studied, with the segment left outside after the surgery to avoid any compression. To maintain hydration, it was covered with a polyethylene film. There was no appreciable benefit with this endeavor (data not shown) and was not utilized any further.

4.2. Final Experiments

4.2.1. Effect of 10 µM AstraNIhn on Pi and fluid a

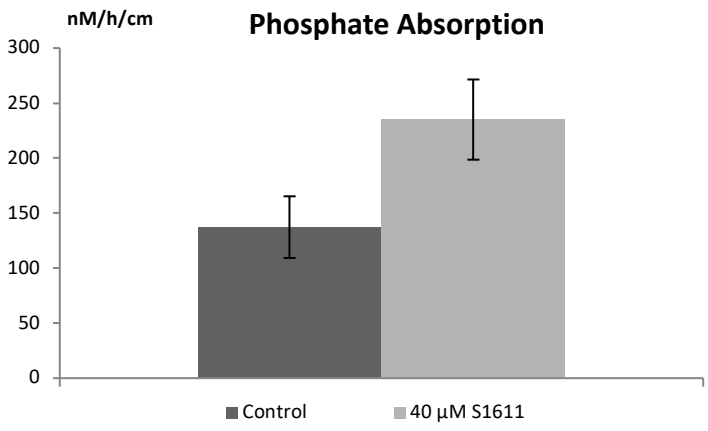


Figure 15 : Pi absorption from the jejunum and effect of 40 µM S1611.

Experiments were done in jejunum for 180 min duration, with addition of 40 µM S1611 at 90 min. First 15 min (at the beginning and after addition of drug) were considered as equilibration period. Fluid absorption was calculated by tracer method and Pi absorption studied with radioactive Pi. The bar graphs represent Pi absorption over 75 min. Pi absorption increased non-significantly with 40 µM S1611, but was low with the low positive calculated fluid flux. (No statistical analysis was performed; n = 3)

Experiments were performed in 7 weeks old rats and a short segment (4 cm) of jejunum was perfused at a speed of ~18 mL/h for a total duration of 150 min. The perfusion solution was HEPES buffered to a pH of 6.8 and contained ^{32}P (up to 2 µCi/100 mL). The fluid flux was measured by gravimetric method. After 60 min of baseline, 10 µM AstraNIhn was added to the perfusion solution to study its effect. A 15 min equilibration period was taken at the beginning and also after addition of drug. Additionally, metamizol 200 mg/kg was given s.c. to alleviate surgical pain.

There was no appreciable effect of metamizol on blood pressure or temperature regulation (data not shown). Hence, metamizol was continued for the rest of study. Average of fluid absorption was considered for the calculations in the study, as it was more relevant to our question. As seen in Figure 16, there was no appreciable effect of 10 µM AstraNIhn salt on either fluid or Pi absorption. There was though a positive fluid absorption at pH 6.8 with HEPES buffered solution. This signifies no meaningful effect of AstraNIhn at this pH or NHE3 inhibition on either of these parameters. A separate experiment was also repeated in older rats (11 weeks), to exclude any effect of age (data not shown). This however showed similar results. A statistical analysis with age-matched controls did not show any statistical variance, when tested with Student's t-test (data not shown). In the absence of any appreciable effects of the drug, the method was abandoned, and further modifications were tested.

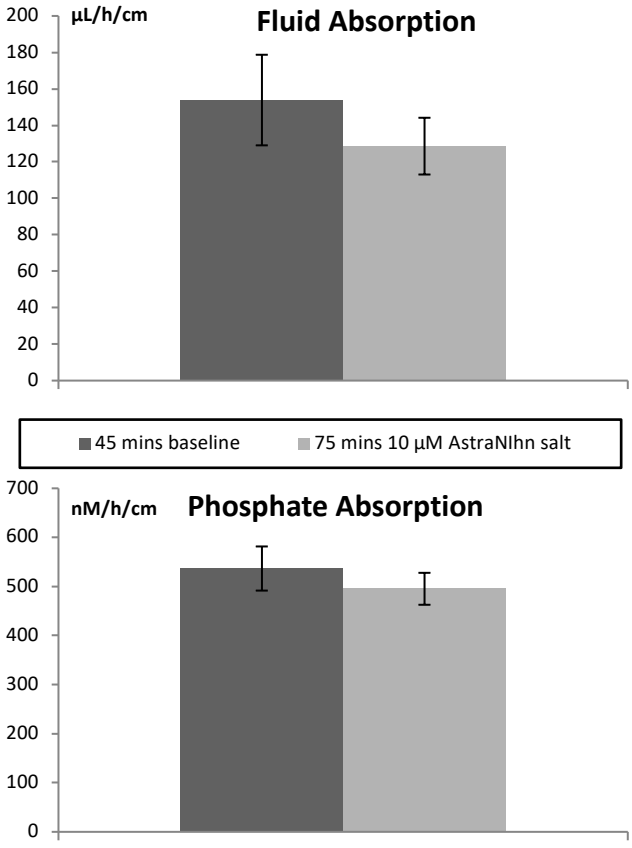


Figure 16 : Fluid and Pi absorption from the jejunum and effect of 10 µM AstraNIhn salt at pH 6.8.

Experiments were done in jejunum for 150 min duration, with addition of 10 µM AstraNIhn salt at 60 min. First 15 min (at the beginning and after addition of drug) were considered as equilibration period. Fluid absorption was calculated by gravimetric method and Pi absorption studied with radioactive Pi. The perfusion solution was HEPES buffered to pH 6.8. Fluid and Pi absorption were not affected with 10 µM AstraNIhn salt. Fluid flux was absorptive at pH 6.8. (No statistical analysis was performed; n = 3)

Experiment was also performed in rats obtained from ZTL, which have lower body weight (appx. 250 g at 8 weeks) than the ones obtained from Janvier labs (350 g), and at a higher perfusion speed of ~55 mL/h. These also showed similar results (data not shown), except for a higher fluid flux seen at the higher perfusion speed (see Figure 17).

In order to prevent any intestinal contraction (which might affect fluid flux), 0.05 mg/kg atropine was given s.c. before anesthesia in a group of rats. In the same experiment, ^{3}H-PEG (up to 2 µCi/100 mL) was also added to perfusion solution in order to study fluid flux by both of the available methods. There was slight increase in blood pressure (up to 140 mmHg) during surgical and recovery period, with atropine. The blood pressure however returned to normal levels during the experiment (90 – 120 mmHg; data not shown). There was similarly no effect of AstraNIhn salt on any observed parameters, but the fluid flux measured with tracer method was much lower (15.43 ± 33.35 µL/cm/h) than the gravimetric method (192.75 ± 56.13 µL/cm/h) (see Figure 17). This shows the limitations of tracer method with incorrect measurements.

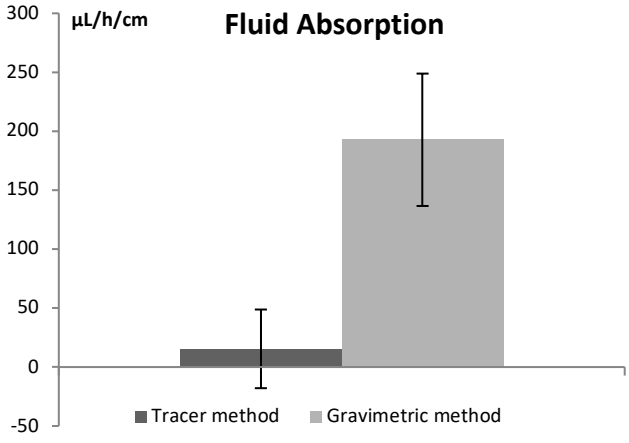


Figure 17 : Comparison of fluid flux in the jejunum measured by tracer method and gravimetric method at pH 6.8.

Fluid flux was measured in jejunum for control duration of 45 min. First 15 min were considered as equilibration period. Flux was calculated by both tracer and gravimetric method. The perfusion solution was HEPES buffered to pH 6.8. Fluid flux was very low to secretive with tracer method, but was absorptive with gravimetric method. P = 0.09 (No statistical analysis was performed; n = 4)

At this point of time, additionally, it was possible for us to compare the effect of the solution and pH on fluid and Pi absorption. Results from rats with similar age and weight were pooled to be compared under different conditions. With different perfusion solutions: i.e. bicarbonate buffered to pH 7.4 and HEPES buffered to pH 6.8 were analyzed for differences in fluid and Pi absorption, when perfused at a speed of ~18 mL/h. In these groups of rats, no additional drug (e.g. atropine) was injected and fluid flux was calculated by gravimetric method.

As it can be seen in Figure 18, the fluid flux measured at pH 7.4 and pH 6.8 were very similar and around 100 µL/cm/h. But, again this data is not very conclusive with a different composition of both perfusion solutions. Bicarbonate containing fluid at a higher pH would normally have higher absorptive rate in jejunum.[66] Though, in our case, bicarbonate

buffered solution at pH 7.4 did not show a higher fluid absorptive flux than HEPES buffered solution at pH 6.8. Similarly, there was no difference in Pi absorption (data not shown).

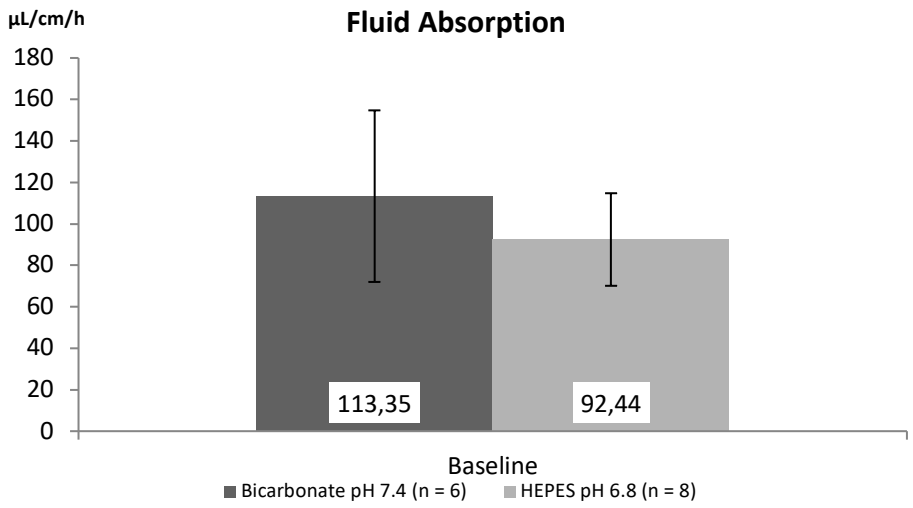


Figure 18 : Comparison of fluid flux in the jejunum: data pooled and analyzed when measured by gravimetric method with different solutions at pH 6.8 and 7.4.

Fluid flux data was measured in jejunum with bicarbonate buffered (at pH 7.4) or HEPES buffered (at pH 6.8) solutions. Flux was calculated by gravimetric method, under similar conditions. Fluid flux was very similar with both solutions and at both pH. Statistical analysis done with paired Student's t-tests; * when $P < 0.05$; $n = 6$.

At this point of time in this study, we obtained another congener of the AstraNIhn drug, in its base form, which has a better solubility at higher pH. In our examination, it was completely soluble at pH 7.4 and hence was utilized in later experiments.

4.2.2. Effect of 10 µM AstraNIhn base on fluid absorption and jejunal contractility

At this point of time, we wanted to study the effect of 10 µM AstraNIhn base on the fluid absorption in jejunum at a pH of 7.4, with a perfusion solution containing bicarbonate buffer. The salt had no appreciable effect at pH 6.8, and the results at higher pH could not be utilized, as the salt congener was not soluble at this pH. We also wanted to test the effect of AstraNIhn base in the presence of a peptide in perfusion solution. As discussed above, peptide transport indirectly stimulates the activity of NHE3 transporter in the enterocytes.[51] So, two set of experiments were performed with bicarbonate buffered perfusion solution at pH 7.4, with or without 1 mM Gly-Sar dipeptide. The peptide containing solutions were always freshly prepared. In both experiments, 10 µM AstraNIhn was added to the perfusion solution after a 60-min baseline. Also, in order to exclude any

effect of varying Na^+ concentration, its concentration was fixed to 100 mM; and the osmolarity of solution was brought to 310 mOsm with mannitol. Apart, the intestinal contraction was recorded as described before. The speed of perfusion was maintained at ~18 mL/h in a 10 cm long segment of jejunum. Also, Pi was removed from the solution to study the role of NHE3 in fluid absorption in the absence of any nutrient and its stimulation in the presence of additional dipeptide. Fluid absorption was measured by gravimetric method.

In 4 rats, the experiment was performed in the absence of dipeptide and showed almost no fluid flux during baseline perfusion. After the addition of AstraNIhn, there was actually an increase in fluid flux to absorption, which was not appreciably variable from the baseline values. It was still much lower than the fluid absorption seen in previous experiments. In another 5 rats, dipeptide was added to the solution during the whole experiment. Here, the baseline absorption was slightly, and not significantly higher than during the absence of dipeptide. This shows a slight stimulation of fluid absorption in the presence of dipeptide. With the addition of the drug however, there was high variance of fluid flux measured in different rats (see Figure 19). These results (again) show an ambiguity in the fluid flux measurements done in our study.

Another 3 rats studied previously to see the effect of AstraNIhn base on fluid and Pi absorption had much higher baseline fluid flux with almost no changes in both fluid or Pi absorption with the drug (data not shown). These experiments were however conducted in different experimental parameters, including a higher concentration of Na^+ ion. All these variance in fluid flux clearly show a high dependence of normal fluid absorption on the concentration of Na^+ and Pi, length of the segment studied and the speed of perfusion, in our study model (discussed later).

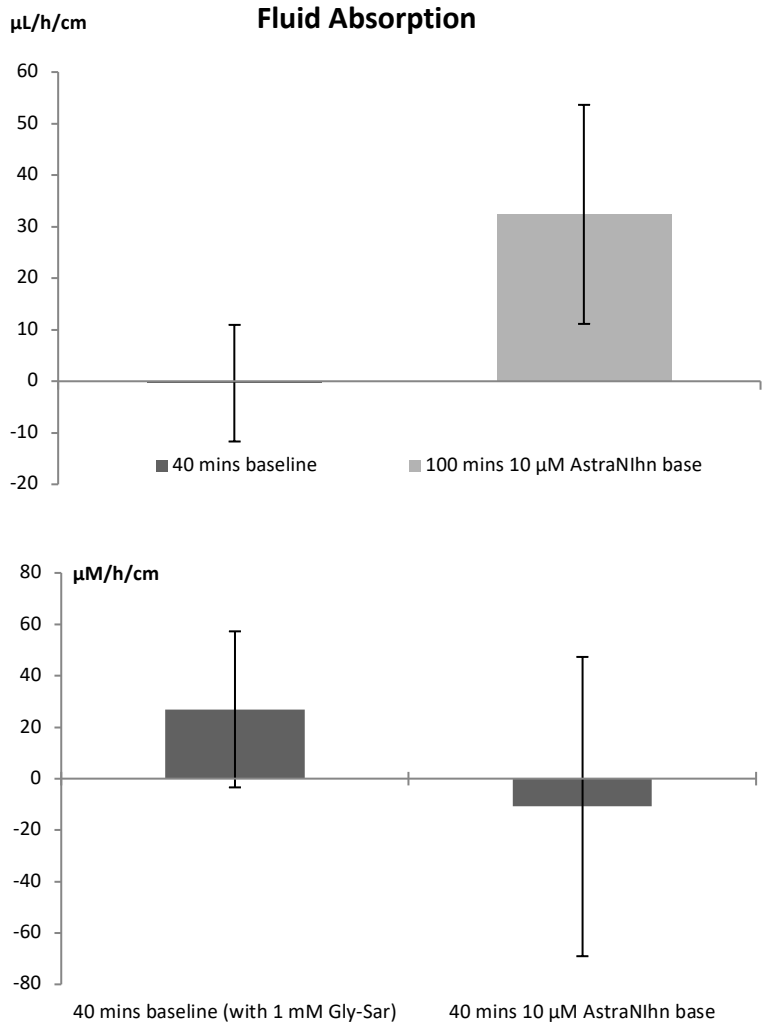


Figure 19 : Fluid flux measured in 10 cm jejunum perfused with bicarbonate buffered solution at pH 7.4, with or without 1 mM Gly-Sar; and the effect of 10 µM AstraNIhn.

Fluid flux was measured in jejunum with bicarbonate buffered (at pH 7.4) solutions. Fluid flux was calculated by gravimetric method. 10 cm long jejunum was perfused at a speed of ~18 mL/h, and Pi was removed from the solution. 10 µM AstraNIhn was added after 60 min baseline perfusion. Upper graph: Perfusion solution contained no peptides. There is small, insignificant increase in fluid absorption after addition of the drug. (n = 4) Lower graph: Experiment was performed with 1 mM Gly-Sar in the perfusion solution. There is a slight but insignificant increase in baseline fluid flux with addition of the peptide. The effect of AstraNIhn on fluid flux is hard to tell. (Statistical analysis done with paired Student's t-tests; * when $P < 0.05$, ** when $P < 0.01$; n = 5)

There was however no effect of 10 µM AstraNIhn base on the blood pH and blood pressure, when measured for duration of 2h after addition of the drug. We also tried to measure intestinal contraction and any effect of AstraNIhn on baseline motility, with an indirect

measurement of intra-luminal pressure. We saw, in few cases, an increase in intra-luminal pressure after addition of the drug (see Figure 20). The changes in intra-luminal pressure, as seen in our case, were probably due to an increased muscle tonus rather than a real peristalsis. With normal motility changes, there would be a rapid contraction with the return of intra-luminal pressure to normal within a short time. Changes in intra-luminal pressure could also be caused by temporary blockage with mucus or movement of tubes. After surgical handling, there is a normal presence of paralytic ileus in intestine. As reported by the group of Nylander, O. *et al*., this temporary inhibition in intestinal motility can be de-inhibited with the use of a COX-2 inhibitor.[67] This however could not be utilized in our study due to restrictions in our experimental permission and the time limit.

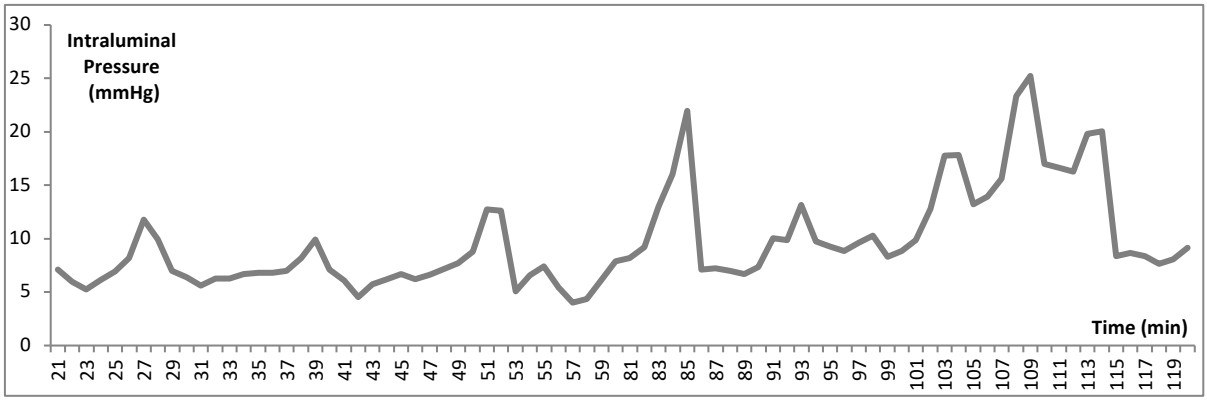


Figure 20 : Intra-luminal pressure measured in 10 cm jejunum perfused with bicarbonate buffered solution at pH 7.4, and the effect of 10 µM AstraNIhn base (single representative trace).

Intra-luminal pressure was measured in jejunum with bicarbonate buffered (at pH 7.4) solutions. 10 cm long jejunum was perfused at a speed of ~18 mL/h. 10 µM AstraNIhn was added after 60 min baseline perfusion. The result of intra-luminal pressure changes were not satisfactory and probably represent an increase in muscle tonus, rather than a real peristalsis. (Shown result n = 1)

4.2.3. Effect of 10 µM AstraNIhn base on fluid absorption in ileum

In this experiment, 10 cm long segment of ileum (starting close to its termination into cecum) was perfused with bicarbonate buffered solution at pH 7.4. The direction of perfusion was physiological and was maintained at ~18 mL/h. The solutions used were similar to the experiment before and had 100 mM Na^+, no Pi and the concentration brought

to 310 mOsm with mannitol. After baseline perfusion of 60 min, 10 µM AstraNIhn base was added to the perfusion solution. Fluid flux was measured by gravimetric method.

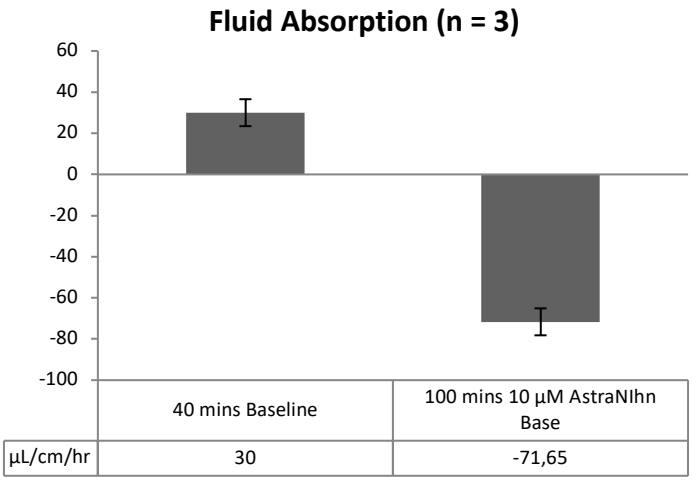
As seen in Figure 21, there was 30 ± 20.49 µL/cm/h amount of fluid absorption seen during 40 min of calculated baseline perfusion and decreased significantly to -71.65 ± 6.55 µL/cm/h, i.e. fluid secretion, with addition of 10 µM AstraNIhn base (P value = 0.031, n = 3). However, there was high rate of complication seen in this group of experiment with the use of drug. Out of 8 rats studied, 2 died ~1 h after the addition of the drug and 2 rats had acidotic blood-gas measurements (e.g. one had a blood pH of 7.13, pCO_2 36 mmHg, pO_2 76 mmHg, bicarbonate 12 mM/L and sO_2 91%), and one rat had very low and seemingly dys-rhythmic blood pressure trace. In both cases of death, infusion of 1 mL of 7% bovine albumin, flushing of tracheal tube with 24 mM bicarbonate containing solution and finally cardiac massage had no survival benefit. This rate of mortality is very high in our setup, while we had very low mortality after a successful anesthesia and surgery (only 2 rats out of previous 62, i.e. <2%). The other 4 rats had normal maintenance of blood pressure and blood-gas parameters (data not shown). Similar complication was however not seen in previous experiments in jejunum. But, this raises a question about possible side-effect of AstraNIhn, which needs to be answered. This could be due to excessive fluid loss or some neural stimulation, as the drug is not systemically absorbed. However, this remains unclear.

Figure 21 : Fluid flux measured in 10 cm ileum perfused with bicarbonate buffered solution at pH 7.4, and the effect of 10 µM AstraNIhn base.

Fluid flux was measured in ileum with bicarbonate buffered (at pH 7.4) solutions. 10 cm long segment was perfused at a speed of ~18 mL/h. 10 µM AstraNIhn was added after 60 min baseline perfusion. There was a statistically significant (P = 0.031) fall in fluid flux, as a secretory response, with the drug. (No statistical analysis was performed; n = 3)

omach

ne tubing (Silastic, Dow Corning, Midland, MI, USA; mouth of the rat and gently advanced into the The pylorus was closed with two ligatures 2-5 mm n after the end of surgery, a solution containing 6 pH 4.6, was given as bolus in 9 weeks old rats. The e silicone tubing was slowly retracted and removed



to allow the closure of lower esophageal sphincter and avoid any reflux. Serum (200 µL) and urine (100 µL) samples were collected immediately before the commencement of experiment and then every 30 min. Blood and urine samples were taken for a total duration of 90 min.

A total of 3 rats were studied for absorption of phosphate from the stomach. After surgery, the stomach was found to be full, and not distended. There was only background level of count detection from serum and urine samples until 90 min after the bolus (data not shown). It suggests no significant absorption of phosphate from the stomach in rats.

5. Discussion

5.1. Gastrointestinal Pi absorption is not completely understood.

As it is with many transport processes, the absorption of Pi is regulated by interplay of different transport modalities, but the contribution made by passive and active processes is not clear. It is difficult to conclude anything with the published data, where investigators have tried to answer only a part of the question. Studies performed in a small segment of the intestine might not translate into the whole animal.[36] And these studies classically ignore the largest segment of intestine, i.e. the ileum, where the passive component would dominate.[24] Pi absorption after a bolus/meal in conscious animals comes closest to the physiology, but is not very suitable for understanding the kinetics of Pi absorption. Also the concentration of absorbable Pi is progressively lower in the distal segments in this model, which however is actually more physiological. The surgical manipulation associated with various models could also affect the Pi transport and result in incorrect inferences. Supporting this, there was an apparent 86% decrease in active glucose absorption with surgical manipulation, in a study involving glucose absorption.[58]

The *ex vivo* membrane model (like Ussing chamber or everted sac) for study of transport processes would theoretically provide a better distinction between different modes of absorption but carry their own limitations. In these models, the neuro-humoral regulation is lost and the transport process can only represent initial kinetics, and not the steady state of the absorption.[58] The other challenge lies with the effect of extracellular pH on Pi transport. The concentration of ionized versus non-ionized Pi and also the ratio of monovalent to divalent form of Pi anion depends very much on the pH. So much so that, the ratio of HPO_4^{2-} (the preferred form of NaPi IIb transporters) falls from ~80% to ~20%, with a pH change from 7.4 to 6.2.[32] Moreover, in the absence of an understanding of the preferred transport form of Pi in passive transport, it is difficult to understand the changes in total Pi absorption with pH. Signifying this, there were reports of increase in total Pi transport both with fall and rise of pH from 7.4,[68, 69] which could be due to a compensatory increase in one transport mode with the decrease in another. In the presence of this ambiguity, it is difficult to estimate the changes in Pi transport, with simultaneous changes in the concentration of transportable Pi.

5.2. Our approach and Findings

We tried to answer this question in an indirect way: i.e. by looking for changes in total Pi absorption with a blockage of intestinal NHE3 transporter, which, though not exclusively, helped us answer its clinical benefits. As discussed before, NHE3 could affect Pi transport by a multitude of pathways. We also utilized this model in an intestinal segment expressing both NaPi IIb and NHE3, which actually can clarify a role of NHE3 in both active and passive Pi absorption. But, this approach was impaired by an absence of any detectable signs of NHE3 inhibition in our study. Apart from this, our study model has other limitations, which are discussed later.

We employed an *in vivo* fluid perfusion model in rat to study the effect of NHE3 inhibition on fluid flux and Pi absorption. This model has been previously and being continually used in our group and many other groups to study different intestinal transport processes.[64, 70, 71] The major role intestinal NHE3 is the absorption of salt and water.[45] We employed the consequent changes in fluid flux, with an inhibition of NHE3, as a determinant of this inhibition. This change in fluid flux is also employed for an indirect measurement of Pi absorption, the resulting calculations of which would be directly affected by changes in fluid flux and any changes in the intra-luminal fluid volume (see above). We, however, did not see any apparent changes in the fluid flux, which was mirrored in the calculated Pi absorption. This questions our whole model, but also questions the role of jejunal NHE3 in fluid and Pi absorption. The apparent indifference in fluid flux could be due to an inactivity of NHE3, an incomplete inhibition by the drug, an incomplete penetration of the utilized drug to the target or a compensatory activation of another transport molecule (see below). Although fluid flux has been awarded a seminal importance in our study, it would be wrong to translate an apparent absence of changes in fluid flux as a complete absence of any changes in Pi absorption.

There was a positive jejunal Pi absorption in our study. The absorbed Pi could also be detected in blood and urine, which rules out a mere adsorption of Pi onto mucosa or absorption and retention in the enterocytes, when measured with our indirect method. The absorbed Pi was transported through the intestinal epithelium and into the bloodstream. There was, however, no detectable decrease in Pi absorption with the application of NHE3 inhibitors, like S1611 or AstraNIhn. There were actually no apparent changes in the Pi absorption, except with the application of 40 µM S1611, which resulted in an increase. This non-significant increase in Pi absorption cannot be explained with an inhibition of intestinal fluid flux, which showed no changes. This could result from renal inhibition of NHE3 and a consequent acidosis, which could stimulate the GI Pi absorption.[63] This result, however, hints towards a possible independence of calculated Pi absorption from fluid flux, in our

study. The detected amount of Pi in blood was very low (~0.3% to the infusate), but the Pi detected in urine was much higher (up to 70%), after 60 min of 2 mM Pi perfusion. With such low counts of detected Pi in blood (probably due to a high dilution and quick excretion), it is difficult to detect changes in Pi absorption. In our experiments, there were no apparent changes in blood Pi count. There was, however, a decrease in Pi excretion in urine with the use of S1611, but not with AstraNIhn. This could have resulted from renal inhibition of NHE3 with S1611. There were also no visible changes in Pi absorption with changes in the perfusion solution and their pH. But with this result, we cannot conclude absences of changes in Pi absorption with pH, as composition of the solution were also changed. In the absence of reliable data on normal intra-luminal Pi concentration, we used 2 mM Pi in all our intestinal experiments. This is close to the intra-luminal concentration, reported by Kirchner *et al*, in weaning rats and was also close to the Km of saturable component.[30] The weaning rats, however, have a much higher expression of NaPi IIb[30] and we expected a smaller contribution of active transport in ~8 weeks old rats, used in our study. At this concentration, we assumed contributions of both active and passive transport processes. In the absence of kinetics data for active and passive Pi transport, it is hard to conclude if one of these was inhibited in our study, with a compensatory increase in the other. But as central to our goal, there was no change in total Pi absorption and hence no benefit of NHE3 inhibitor drugs could be ascertained. Also, we saw no absorption of Pi from the stomach at concentration as high as 10 mM.

We primarily utilized the upper segments of intestine for our study. Jejunum in rats express both NaPi IIb and NHE3.[31, 53] This was done to answer our primary question, as whether the beneficial effects of NHE3 inhibition on Pi homeostasis, as reported by Ardelyx Inc.[46, 48], is due to a direct role of NHE3 transporters on Pi absorption in jejunum. As a segment expressing the active transporters, it is expected to be responsible for the majority of active Pi uptake from GI tract. A decreased Pi absorption in this segment would have signified a direct role of NHE3 transporters in active and/or passive Pi transport. Though with our study, this cannot be ruled out. It is also possible that the effects seen by Ardelyx Inc. are due to an effect of NHE3 inhibition on Pi absorption in distal segments of intestine. Due to the absence of any observable NHE3 inhibition, in the form of fluid flux or Pi flux, our second goal could not be answered.

5.3. Role of NHE3 in rat jejunum

The Na^+/H^+ exchanger isoform 3 (NHE3) was central to our study. Its role in fluid absorption and in the regulation of other transport processes is well recognized and is known to be expressed in the whole length of intestine (as described above in details). But, its role in GI Pi absorption is questionable. As reported by Ardelyx Inc., intestinal NHE3 inhibitors decreased the total Pi absorption from intestine and were also effective in decreasing the end-organ damage.[46, 48] But, this finding doesn't signify a direct role of NHE3 in Pi transport. Such an effect could also be caused by a simple increase in GI motility and a decreased transit time, which itself is probably an indirect effect of the decreased fluid absorption. Also, such an effect might not be permanent and that questions its effectiveness in long term, especially in a chronic condition like CKD. For example, the expression and activity ENaC channels (Epithelial Na^+ Channel) increases in NHE3 KO mice, which could compensate for the decrease in fluid and Na^+ absorption seen in these mice.[50] Regardless of this, in our model of *in vivo* perfusion in rat jejunum, we did not see any significant changes in fluid absorption with intestinal NHE3 inhibitor. Also, there were no important changes in fluid absorption with changes in intra-luminal pH. There was, however, differences in fluid flux with a change in the composition of perfusion solution and the perfusion speed. This apparent absence could be described based on an inactivity of NHE3 channels or the inability of AstraNIhn to block it. There was however, a significant decrease in fluid absorption with AstraNIhn in ileum. This clearly proves an inhibitory role of AstraNIhn. Also, there were no changes in blood pH or blood pressure, unlike as described by Ardelyx Inc.[46] or as reported in NHE3 KO mice.[50] Such a small change could have been easily compensated in our short term study. It could also be due to the short length of intestine tested, and hence lesser amount of NHE3 was blocked.

In our study, AstraNIhn had no inhibitory effect on fluid absorption in rat jejunum. But in other studies conducted in our group, we found an inhibitory effect of AstraNIhn on fluid absorption and also a decrease in electrical flux due to peptide absorption in mouse jejunum, in *in vivo* perfusion model and in Ussing chamber respectively (see Figure 22). In the first result performed in *in vivo* perfusion model with mouse jejunum, there was significant increase in fluid absorption with the simple addition of bicarbonate buffer in the perfusion solution. This increase in fluid absorption in the presence of bicarbonate has been previously reported[66], but was not seen in our rat model. Also, there was a significant decrease in fluid absorption with 1 µM AstraNIhn base, both in the presence and absence of bicarbonate. Again, with the Ussing chamber study, there was an expected bipashic response in short-circuit current with the addition of 20 mM Gly-Sar in the luminal solution. The second part of this biphasic response is probably due to an activation of NHE3 channels

in response to peptide transport, and the consequent increase in total peptide transport. With the addition of 10 µM AstraNIhn base to the same side, there was a significant return of this response towards the resting value. Again, this is probably due to a subseuent inactivation of NHE3 and the consequent decrease in total peptide transport. Both of these results clearly showed that the NHE3 activity was inhibited with AstraNIhn base in mouse jejunum, and in case of *in vivo* perfusion experiment, the inhibition was already significant with 1 µM concentration of the drug. Apart from the inhibition of bicarbonate and peptide stimulated activity of NHE3, the basal fluid flux was much lower when perfused with iso-osmolar saline. This clearly shows that NHE3 is basally active and contributes to salt and water absorption in mouse jejunum. All these results also make it clear that the absence of any fluid flux change in rat jejunum is species specific.

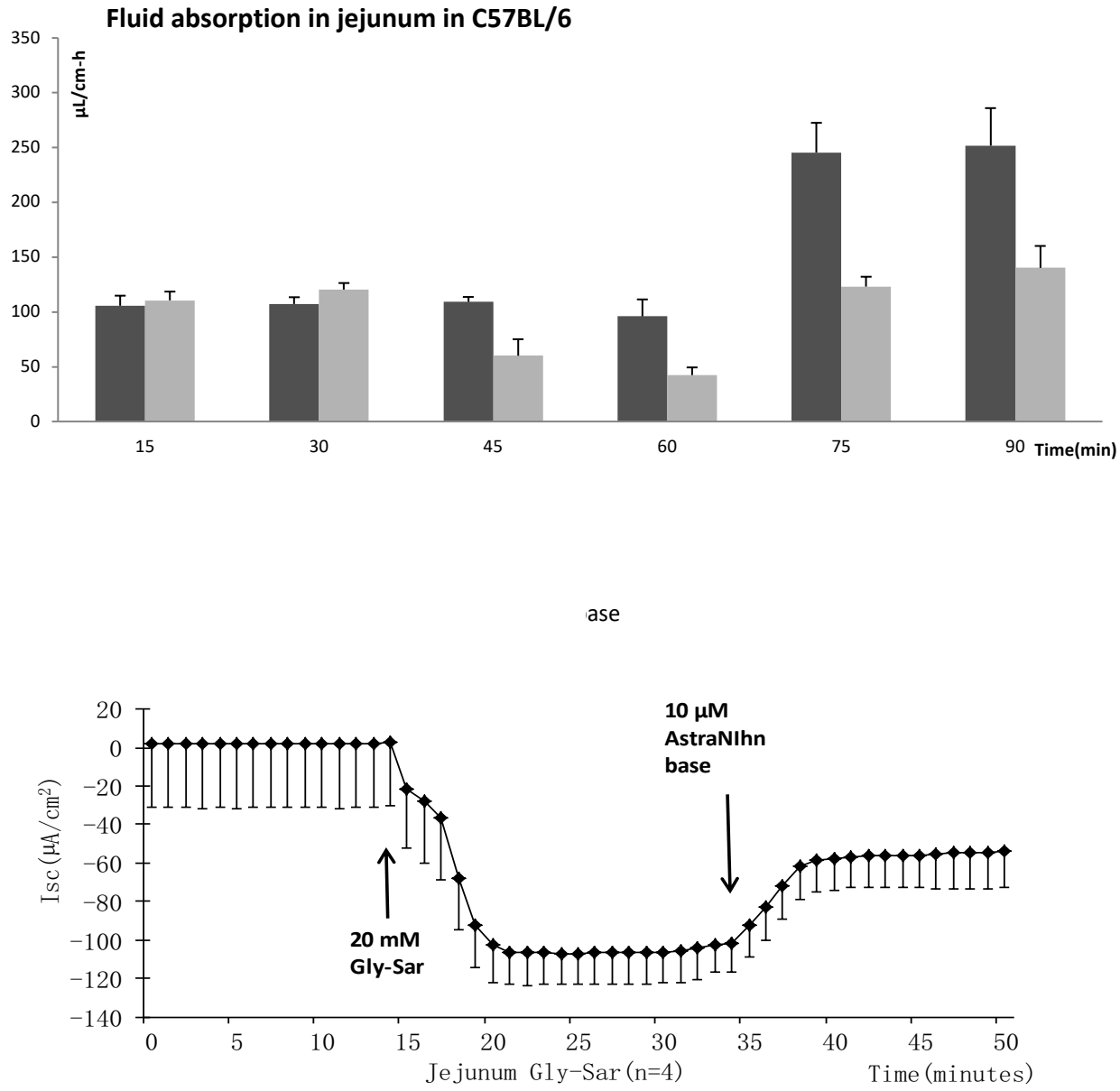


Figure 22 : (Upper Figure): Changes in fluid flux in mouse jejunum with an addition of bicarbonate buffer and then 10 µM AstraNIhn base and (Lower Figure): changes in short-circuit current response (Isc) in mouse jejunum with addition of 20 mM Gly-Sar and then 10 µM AstraNIhn base.

Upper diagram: Fluid flux was measured in *in vivo* perfusion model in mouse jejunum. For first 30 min, it was perfused with normal saline and showed ~100 µL/cm/h fluid absorption. After 30 min, 10 µM AstraNIhn base was added to the treatment group and continued for the rest of experiment. In both control and treatment group, the solution was changed to bicarbonate buffered saline at 1h, which showed a significant increase in fluid absorption irrespective of addition of the drug. 10 µM AstraNIhn inhibited fluid absorption significantly when perfused with both unbuffered and buffered saline solution. (n = 4, Statistical analysis done with paired Student's t-tests; * when $P < 0.05$, ** when $P < 0.01$; *Courtesy: Jiajie Qian*)

Lower diagram: Short-circuit current response (Isc) measured with mouse jejunum in Ussing chamber. There was a significant biphasic response with addition of 20 mM Gly-Sar on the luminal side. Afterwards, there was a significant return of this response towards baseline with addition of 10 µM AstraNIhn base in the luminal side. (n = 4, Statistical analysis done with paired Student's t-tests; * when $P < 0.05$; *Courtesy: Yongjian Liu*)

So, is NHE3 not active or is AstraNIhn not able to inhibit NHE3 in rat jejunum? It is a difficult to answer this question with our study. NHE3 is expressed in rat jejunum and this has been reported with both histological[49] and functional studies[45]. But, the basal activity of NHE3 in rat jejunum is highly questionable. The activity of NHE3 is pH dependent, with a higher activity seen with a higher intra-luminal pH.[45, 72] The intra-luminal pH in jejunum is normally around neutral, which does not justify this inactivity. But, the epithelial brush border membrane is remarkably covered with an unstirred layer, constituted mainly of mucus. This layer is generally acidic in jejunum, with a pH varying from 6.1-6.8 in normal conditions; and this micromileau is widely known as the acid micro-climate (ACM).[51] It is, however, this micro-environment where all the membrane transporters are required to function. Also, at this pH it is possible that NHE3 is basally inactive.[33] There are reports suggesting a role of NHE3 in maintenance of this ACM in the first place.[52] But, this role cannot be played by NHE3 alone and contributions of NHE2 is also reported.[52] It is also possible that NHE3 is partially activated during basal conditions, which is reported to be inhibited early in digestive phase, with a later activation.[45] This might play important roles in the completion of digestion in upper parts of intestine. After food intake, there is also an intra-luminal acidification in the upper small intestine.[73] The physiological peptide absorption is known to be more efficient during acidic pH.[74] Initial peptide absorption could stimulate NHE3 and hence a circulation of H^+ ion: in a process to remove it from enterocytes and make it available in the lumen for the peptide absorption. The ACM is maintained in our model of rat and it is therefore important for us to consider its role. But in our case, the perfusion solution always had a high buffer capacity, which would prevent any changed in intra-luminal pH. However, even with perfusion solutions at pH 7.4, the ACM pH would not be necessarily affected. A study reported the maintenance of an acidic ACM pH with perfusion of solution at neutral pH to even alkaline pH. When perfused with solution at neutral pH, the ACM pH remained towards 6.3, but was more acidic with perfusion solutions containing glucose.[33] In the presence of glucose,[56] NHE3 is activated by SGLT1 and this would explain the more acidic acid micro-climate. Again, inhibition of NHE3 with 100 µM EIPA had no effect on fluid absorption in another study.[66] We also had experiments with perfusion solutions containing bicarbonate, which would theoretically increase fluid absorption and also stimulate NHE3.[66] This was, however, again not seen in our case. But, in the above study, there was no change in ACM pH with bicarbonate containing perfusion solutions.[33] In another study, the pH of ACM was found to be 6.25 when the perfusion solution was buffered to a pH of 7.25 with bicarbonate. Also, the inhibition of NHE3 increased the surface pH, but this does not directly imply a role of bicarbonate on the maintenance of ACM pH.[75] To summarize, the activity and the role of NHE3 in jejunal fluid absorption in rats is controversial. The literature suggests an absence of such a role, with a basal contribution in the maintenance of ACM. In the absence of an understanding of basal NHE3 function, it is

complicated and probably not correct to predict a role of its inhibition, with a possible disruption of this micro-mileau, on the intestinal Pi absorption. Finally, in the presence of ACM, there would be a much lower effective concentration of NaPi IIb transportable Pi.[32]

Apart, it is also possible that AstraNIhn is not able to traverse the unstirred layer in rat jejunum, hence is inactive. However, there was also an insignificant changes in jejunal fluid flux with the application of S1611. NHE3 inhibitors could also function by changing the neural firing in the enteric nervous system.[50] These are the important questions to answer, before we could certify a beneficial role to NHE3 inhibitors in the treatment of chronic conditions.

5.4. Limitations of our experimental model

We employed an *in vivo* single-pass perfusion model for our study of membrane Pi transport. We studied the jejunal fluid flux and utilized the same to indirectly calculate the Pi transport. This, like every other technique, has its own limitations. Surgical manipulations and anesthetic drugs can also affect a transport processes by themselves. Surgical handling has been shown to decrease active glucose absorption by as much as 86%.[58] There are open questions on the effects of anesthetic drugs on transporters like NHE3.[55] This could have resulted in the apparent toxicity of NHE3 inhibitor in our ileum experiments, which has not been reported previously. This could be caused either by a loss of effectiveness of drug reaching the ileum, in published studies,[46-48] or due to the presence of an interaction with the anesthetic drug. Irrespective, short term perfusion models carry limitations as study models to study a steady-state transport process. In our model, the salivary and pancreatico-biliary secretions were cut-off from the test segment. In the absence of knowledge of their physiological role in Pi absorption, it is difficult to predict its effect in our model.

The Pi absorption, in our study, was indirectly calculated, based on the measurement of fluid absorption. The measurement of the absorbed Pi in blood requires a high amount of radio-active phosphate in perfusion solutions. This is due to a rapid excretion of absorbed Pi from the kidneys and a dilution in the intra- and the extracellular fluid space. Hence, it is difficult to detect minute changes in Pi absorption with small counts. Apart, our study model had various limitations of its own, sometimes due to complexity of the question asked, as mentioned below:

- **We tested Pi absorption in a short segment of intestine.**

  For our study, we employed a short segment of jejunum to study Pi and fluid absorption and their changes with NHE3 inhibition. We utilized jejunum, as it is the segment with co-expression of NaPi IIb and NHE3 transporters.[31, 53] This would answer our aim to check whether the reported decrease in Pi absorption with NHE3 inhibition[46, 48] occurs due to a direct role of NHE3 in Pi absorption. It is possible that the published effects of NHE3 inhibition occurs in a later segment of intestine and/or affect the passive component of Pi absorption.

- **The fluid absorption observed in our experiments was too low.**

  We used perfusion solutions without any absorbable nutrients to study the basal Pi absorption, determination of which was dependent on fluid flux. In the presence of a nutrient like glucose or peptide, there would be a sufficiently large stimulation of fluid absorption, as seen with the intake of oral rehydration solutions.[65] Such stimulation in fluid absorption can affect the resultant Pi absorption, e.g. with a resulting solvent drag. Although more physiological, this method is unsuitable for an understanding of the process. Reliability of the findings decreases exponentially with such low fluid absorption, as suggested by Madara *et al*.[58] The variance between experiments is also subsequently very high. The small fluid flux could also be caused by high perfusion speed or a short intestinal segment length. But in our experiments, the changes in these criteria did not result in any satisfactory improvements.

- **The fluid flux measured with tracer method is inaccurate.**

  As it is reported to be better in terms of accuracy and reproducibility, we utilized the tracer method for calculation of fluid flux. This however led to significant differences from the fluid flux measured with gravimetric method. The result was a too low absorption to secretion with the utilization of tracer method in our study. This could have arisen from an adsorption or absorption of the tracer onto the epithelium or into the blood-stream respectively. Various reports suggest a detection of PEG 4000 in mesenteric vein[76], an incomplete recovery in the exfusate[66] or an increased absorption with the stimulation of other transporters[73]. This is actually a big issue, as a loss of even 1% of perfused PEG in our model would lead to an under-estimation of fluid flux by ~75 µL/cm/h, in a 10 cm long segment of intestine perfused at a rate of 20 mL/h. The suggested solution to this problem include purification of tracer by gel-

filtration before each use[62] and addition of cold PEG 4000 to decrease the proportionate loss.[77]

- **The fluid flux measured with gravimetric method showed high variability and low reproducibility.**

  The variability of fluid flux with gravimetric method could be due to intestinal contraction or distention, both of which could be caused by the anesthetic drug or the experimental drug. This could also occur due to external pressure, which can bring temporary changes. The seldom extrusion of mucus with the effluent also brings a factor of under-estimation and variability. The reproducibility is low with the low fluid flux in our set-up. The fluid flux, in our experiments, is so low that even a minute delay in sample collection can bring a significant error. So much so that, even a 5 seconds delay in fluid collection would under-estimate the fluid absorption by ~25 µL/cm/h, which is close to 25% of basal fluid absorption seen in our experiments.

- **Apparent dormancy of NHE3 activity.**

  We estimated the NHE3 activity with changes in fluid flux. But as seen in our experiments, there was no significant change in fluid flux. This could be either due to absence of any basal activity of NHE3 or due to compensation of fluid flux with another transporter. It is hard to predict the more probable factor. A major way NHE3 could affect the GI Pi absorption, is by decreasing the transit time of food bolus. This in turn results due to a decreased fluid absorption and the resulting increased motility of intestines. But in our model, the speed of perfusion was clamped, and the average intestinal transit time would not vary, with any such activity of NHE3. Though this could be an important study parameter, it is hard to tell when there are no differences to be seen. Our attempt to study an effect of NHE3 inhibition on intestinal contractility was not successful either. Logically however, there would not be any increased motility resulting indirectly from such decrease in fluid absorption, due to the above mentioned clamped speed of perfusion in our model.

- **Small experimental number and limitations of the statistical analysis:**

  In the majority of "validation experiments", the chosen number of experimental animals was kept low to answer the primary aim of validation of our experimental model. In the absence of sufficient number experimental animals, a statistical analysis could not be performed, and this limits the significance of those experiments. This was also, however, the case in most of our final experiments. The apparent problems and challenges had to be answered with changes in the experimental parameters and hence limited the final experimental numbers. In the absence of an appreciable variance with any particular change, it was subsequently abandoned in order to be able to find an optimal method; limiting the total number of experimental animals. Hence, a statistical analysis could not be performed in many of the final experiments. But in this case, these results have been considered as statistically insignificant. This, however, effectively reduces the statistical strength of this whole study.

Finally there are other limitations in our model, like an absence of distinction between the modes of phosphate transports, decreased effectiveness of pH changes (due to presence of ACM), species differences and the simplicity of experimental model, which might not translate into complex patho-physiologic situations.

5.5. Future Directions

Hyperphosphatemia in CKD is a complex problem, but there is a pressing need for an understanding of this pathology. With this study, the primary questions could not be answered, but this leaves us with an important opportunity to design further experiments. Considering the results, the challenges and the shortcomings of this study, there are many different possibilities one could follow. The first would be an improvement in measurement of the fluid flux, with better implementation of tracer method or by improving the fluid flux. One possible way would be the one suggested by Madara *et al.*, who suggested a multi-pass perfusion model to increase the total amount of fluid absorbed with respect to the volume of fluid perfused.[58] This is an important way to minimize errors and also improves reproducibility. The osmolarity of the perfusate can be artificially fixed during such process, but this technique also carries an important limitation. The concentration of the substrate and/or the drug is progressively lower with increase in the number of passes. But still, this method could solve the important problem of low fluid flux. The other important model to

understand normal physiological Pi absorption would be Ussing chamber (or everted sacs) and the BBMV. Active and passive transport can be easily differentiated with the use of an inhibitor of active transport. Phosphonoformic acid (PFA) is an important candidate for such a role, which is otherwise nephrotoxic and carries limitations in *in vivo* models.[31] Another useful technique would be to give a dose of Pi as a bolus liquid or solid food and study the differential absorption from different segments of intestine, as described in few reports.[24, 36] This model can be important to study the effect of drugs, with a more reliable translation into a treatment. But, this model also carries an important limitation of a progressive decrease in the available Pi concentration, which is however more physiological. Finally, in our model of *in vivo* single pass perfusion, it would be interesting to see the absorbed Pi in blood, when the excretion of absorbed Pi is prevented. This could be surgically accomplished by ligating both kidneys. Though reported to result in no significant changes in blood pressure or blood-gas parameters,[78] this puts another layer of isolation of the experimental segment and its associated unreliability. In the same time, this model brings a great advantage of bypassing limitations of fluid absorption. It would also be interesting to artificially stimulate NHE3 activity, e.g. with a nutrient like glucose, to see if it plays a role in increasing the Pi absorption. Finally, it would be interesting to see the effect of ileal NHE3 inhibition on Pi absorption in ileum. This would clarify a role of NHE3 in passive Pi transport.

Most of the studies to learn the pathology and the potential interventions of CKD are done in some established models of CKD in rodents, which can be generated by subtotal nephrectomy or by treatment with adenine. In both of these models however, it is necessary to accelerate the development of hyperphosphatemia, which is generally achieved with a diet consisting high amounts of phosphate.[79] Though successful in development of hyperphosphatemia, with the related complications and also in the studies of treatment modalities, this model carries important limitations. For example, in these acute models, the loss of function of 1-α hydroxylase is not completely developed.[7] One potential improvement would be in the studies conducted with Cy/+ rats, who develop CKD-MBD more slowly. In Cy/+ rats, no beneficial effects with binder therapy is seen, unlike the faster-developing CKD models; and the levels of various factors involved in Pi homeostasis is much different,[79] which also showed a significant improvement in other models with treatment. Similarly, this slow-development model of CKD had a decreased intestinal Pi flux, while the rapid model of CKD development has consistently reported a normally maintained Pi absorption in CKD.[10, 18, 19] Importantly, this raises a concern about the validity of the reported benefits of NaPi IIb mutation[42] or with NHE3 inhibitor treatment[48], against hyperphosphatemia in rapid models of CKD. Whether these studies can translate into a practical use in patients, is a question.

Finally, it is important for us to learn from other failures and the efforts to overcome them. Transport of glucose was not understood until recently, due to its complexity. In the case of Pi, its regulation and probably also its absorption comes hand-in-hand with calcium. Interestingly, trans-cellular absorption of Ca^{+2} is predominant in duodenum. But paracellular absorption can take place through the whole length of intestine. It sounds similar, in this respect, to the Pi transport modes seen in rats and humans. But, Ca^{+2} absorption is well studied and it is now known that the majority of GI Ca^{+2} absorption takes place in ileum. [80] Again, reports suggest an increase in passive Ca^{+2} flux with the activation of NHE3, which itself is also regulated by vitamin D.[57] Ca^{+2} also plays important roles in homeostasis of Pi and probably also in the pathology of CKD-MBD. It has been shown to be a more potent factor in the release of FGF23, both after GI intake[8] and with its serum concentrations[81], than Pi. It could be actually counterproductive to segregate transport processes of Ca^{+2} and Pi, which are strongly interdependent on each other. To emphasize this issue: the usual models to study a low Pi diet and its effect on Pi transport usually employ a high Ca:P ratio in the diet, which could have their independent effect on their metabolism.[82] Also, the probable roles of NaPi IIb in GI tract are interesting and it also seems to have an important role in Pi homeostasis. It might have a role in the secretion of phosphatonin after Pi intake and resulting in regulation of serum Pi concentration.[10] The presence of external Pi sensing in isolated enterocytes[83] and the quick release of PTH after an intake of Pi, which actually precedes the rise in serum Pi, justify such a role;[84] and NaPi IIb is an excellent candidate for it. It is also possible for it to play a role in the transport of Pi into the enterocytes, for their nutritional/metabolic requirements, and have less important role in the systemic Pi absorption.[30] It is possible that the GI Pi absorption is a simple passenger of the fluid absorption, during normal physiological conditions, which however could also be affected by NHE3 activity. These are important questions to answer for a successful development of a treatment modality against hyperphosphatemia in CKD.

5.6. Conclusions

First observed serendipitously and then reported with an extensive report by Ardelyx Inc., intestinal NHE3 inhibition showed a beneficial effect on Pi homeostasis, when evaluated with respect to CKD hyperphosphatemia.[46, 48] They reported a decrease in GI Pi transport, fall in serum FGF23 and serum Pi counts and also a beneficial effect on end-organ and vascular calcification in a rat model of CKD. But, the mechanism behind such a beneficial effect of NHE3 on Pi homeostasis was not clear. It is equally possible that this effect could just be a short-term side effect in an experimental model of (fast) CKD, which could not be translated into a more slowly developing CKD in patients.[85] It is important for us to understand the mechanism of such an effect before we could prescribe a role to this new class of inhibitor drugs. To examine this, we asked if the observed effect was due to a direct role of NHE3 transporters on Pi absorption in jejunum. As an intestinal segment with expression of both NHE3 and an active Pi transporter, NaPi IIb, jejunum is a potential site for an NHE3 mediated Pi absorption.[31] NHE3 inhibition could also affect the Pi absorption indirectly by its effect on fluid absorption or intestinal motility[45], and such a role might not be long-lasting. In this study, we studied the role of NHE3 inhibitor on Pi and fluid absorption in different segments of intestine. In our study, we did not find any effect of NHE3 inhibition in the jejunum of rats, neither on Pi absorption nor on fluid flux. At different concentrations and with different formulations, there were no significant changes in this segment of intestine. This could be due to an absence of basal activity of NHE3 in this segment or an incomplete penetration of the inhibitor drug through the mucosal layer. There was however, a decrease in fluid flux with the same drug in ileum.

This indicates a possible absence of basal activity of NHE3 in the jejunum of rats and hence absence of a significant role of NHE3 in Pi absorption in this segment of GI tract. The effects seen by Ardelyx Inc. could also be due to a decreased Pi absorption in ileum, due to NHE3 inhibition. With our report, it is still unclear if NHE3 plays a direct role in intestinal Pi absorption or the previously published beneficial effects are just a side-effect of NHE3 inhibition. Future studies are necessary to clarify both hypotheses to better understand the function of NHE3 as well its role in GI Pi absorption.

Summary

Background: Hyperphosphatemia in Chronic Kidney Disease (CKD) often manifests as bone disease and can result in death due to cardio-vascular complications. The effective treatment options are sparse and are associated with side-effects and low compliance. There is a long-searched need for a better drug against this dysregulation in phosphate (Pi) homeostasis. Na^+/H^+ exchanger isoform 3 (NHE3), which is otherwise important for salt and water absorption from intestines, is an interesting target candidate. In a report by Ardelyx Inc., a new class of intestinal specific NHE3 inhibitor drugs showed decrease in GI Pi absorption and also beneficial effect in end-organ calcification. But, the mechanism of this beneficial effect of NHE3 inhibition on decreasing the GI Pi absorption could not be explained. This could be due to a direct role of NHE3 in active and/or passive Pi absorption or due to the side-effects of NHE3 inhibition, such as: a decrease in fluid absorption or an increase in motility, both of which might be temporary.

Aim and Methods: Our aim was to understand the changes in total Pi absorption from intestine, with inhibition of NHE3 in jejunum, which is the segment of intestine expressing both NHE3 and NaPi IIb, an active Pi transporter. In this way, we wanted to assess the effectiveness of NHE3 blockage as a therapeutic target against hyperphosphatemia in CKD. We used an *in vivo* single pass perfusion model to study the normal Pi absorption and the effect of NHE3 inhibition on it. We selected rat as the experimental species and the jejunum as the experimental segment, as it is probably the most important segment involved in regulated Pi absorption. We also studied the changes in fluid absorption with NHE3 inhibition in jejunum and ileum, as a parameter for NHE3 activity.

Results: The fluid absorption and Pi absorption did not change appreciably with the use of an intestinal specific NHE3 inhibitor in jejunum, with varying experimental conditions, such as the changes in: luminal pH, length of the experimental segment and other experimental parameters. Also, with a non-specific NHE3 inhibitor, there was no change in fluid absorption from jejunum, while the total Pi absorption increased slightly, with a higher dose. The fluid absorption decreased significantly with the intestinal specific NHE3 inhibitor in ileum but showed other side effects. There was absence of any Pi absorption from the stomach.

Conclusions: The absence of changes in fluid flux with NHE3 inhibitor could be due to an absence of basal activity of NHE3 in jejunum. This could also be due to an incomplete penetration of the drug through the mucus layer. There was also an absence of any effect of Pi absorption. The beneficial effects seen by other groups could also be due to a role of NHE3 in Pi absorption in other segments of intestine.

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Eigenständigkeiterklärung

Erklärung nach § 2 Abs. 2 Nrn. 6 und 7 der Promotionsordnung der Medizinischen Hochschule Hannover

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel „Role of Sodium/Hydrogen Exchanger Isoform NHE3 in Gastrointestinal Phosphate Absorption“ in der Klinik für Gastroenterologie, Hepatologie und Endokrinologie unter Betreuung von Prof. Dr. med. Ursula Seidler mit der Unterstützung durch Dr. Markus Sjöblom, Uppsala Üniversität, Schweden und Dr. Brigitte Riederer und in Zusammenarbeit mit Yoko Narasaki, Tokushima University, Japan ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe.

Ergebnisse der Dissertation werden in keinem Publikationsorgan veröffentlicht.

Hannover, den 21.03.2018

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(Alok Garg)