Potential Contributors To The Decreased Vitamin B6 Levels After Transplantation

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Vitamin B6 deficiency as measured by plasma concentrations of pyridoxal 5` phosphate (P5P), the active form of vitamin B6, has been reported in several organ transplant recipients. The highest incidence of vitamin B6 deficiency has been observed in small bowel transplant recipients. The current study evaluated the potential mechanisms (increased degradation of P5P due to higher levels of alkaline phosphatases; or decreased formation of P5P due to decreased expression and activity of pyridoxal kinase) that may contribute to such observations. Higher plasma concentrations of alkaline phosphatases, decreased plasma albumin concentrations and higher amount of excretion of 4-pyridoxic acid in the urine of small bowel transplant patients supported the role of increased degradation of P5P. Of the various medications used by transplant patients that were tested using computation approach, pantoprazole, trimethoprim, acyclovir, valganciclovir and mycophenolic acid appeared to be most likely to inhibit pyridoxal kinase. Tacrolimus, the primary immunosuppressive drug used in most transplant recipients had limited impact on pyridoxal kinase expression and activity. A pro-inflammatory cytokine, TNF-α, decreased the expression and activity of pyridoxal kinase in primary cultures of human hepatocytes. The higher plasma concentrations of TNF-α observed in small bowel transplant patients is consistent with a decreased activity of pyridoxal kinase in the liver of these patients, and supported decreased formation of P5P also as a contributor to the observed vitamin B6 deficiency. Additional studies in an animal model where an organ that was not directly involved in the absorption, formation or degradation of vitamin B6 (a composite allograft tissue

transplantation), showed a significant reduction in the expression of pyridoxal kinase in the liver. This suggested that the process of organ transplantation (immune activation and inflammation) itself led to vitamin B6 deficiency. Our studies indicate that vitamin B6 deficiency is expected in all transplant recipients; however, the magnitude of deficiency is likely to be affected by the immunogenicity of the transplanted organ and the immune status of the patient. Given the potential effect of vitamin B6 deficiency (neurotoxicity), and the low cost of vitamin B6, it is prudent to consider supplementation of all transplant patients with vitamin B6.

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1.0 INTRODUCTION

Organ transplantation has become the treatment of choice for many patients with end stage organ failure. Organs such as kidney, liver, lung, heart, pancreas, and small intestine are routinely transplanted and organ transplantation has significantly improved the over all patient survival. However, despite the relative success of this treatment option in terms of decreased morbidity and mortality, post-transplant care has continues to be a challenge. These care plans include the use of immunosuppressant medications, and prophylactic medications (antifungals, antivirals, etc.), in addition to the medications administered for existing other co morbidities (diabetes, hypertension, hyperlipidemia, etc.). Besides these treatment protocols, the functional recovery and the nutritional status are critically important determinants of the overall success of transplantation. For example, clinical nutritional autonomy is usually achieved shortly after small bowel transplantation in most cases. However, the full functional recovery of the transplanted small bowel has not yet been completely evaluated. In a recent study by Matarese *et al.*, a full scale screening of serum concentrations of micronutrients in intestinal transplant patients revealed a significant deficiency in vitamin B6. The study identified specific risk factors and established a clinically useful repletion protocol for small bowel transplant patients.

Normally, vitamin B6 deficiency is rare and it does not usually progress towards a clinical deficiency that necessitates an intervention. This is due to the fact that vitamin B6 is widely available through diet and is also synthesized by the colonic micro flora. Various explanations have been offered to account for the decrease in plasma P5P concentrations in majority of small bowel transplant patients, including reduced dietary intake and/or poor intestinal absorption of vitamin B-6, increased degradation by alkaline phosphatase (ALP), altered metabolism of vitamin B-6, increased urinary excretion of 4-pyridoxic acid (4-PA) and drug-nutrient interactions. Deficiency of vitamin B6 following small bowel/multivisceral transplantation may also be due to increased metabolic demand due to trauma and inflammation. The increasing metabolic demand due to surgery or inflammation can not adequately explain the development and progression of vitamin B6 deficiency several months after transplantation. Similarly, the fact that Vitamin B6 is mainly absorbed by passive process and that the deficiency is reported in various other solid organ transplant recipients makes poor intestinal absorption a less likely explanation for the observed vitamin B6 deficiency in transplant patients. On the other hand, increased degradation of pyridoxal 5` phosphate by enzymes like alkaline phosphatase into pyridoxal, which is ultimately catabolized to urinary 4-pyridoxic acid or the impact of drugs taken by transplant patients on pyridoxal kinase, the enzyme responsible for formation of pyridoxal 5` phosphate, has not yet been investigated.

1.1 VITAMIN B6

Vitamin B6 is one of the essential vitamins that the human body is not able to synthesize. It exists in various chemical forms that differ from each other in the substitutions of different groups at position 4. Pyridoxine carries a hydroxyl group, pyridoxal an aldehyde and pyridoxamine an amine group at this location [1]. Vitamin B6 is a water soluble vitamin that is passively absorbed from the upper part of the small intestine by simple diffusion [2]. Vitamin B6 is obtained from two different sources: in dietary source, from which it is mainly absorbed in the small intestine passively, and bacterial source, where the vitamin is synthesized by the micro flora in the large intestine. A carrier mediated active transport was suggested to be involved in the uptake of micro flora synthesized vitamin B6 [3]. The intestinal absorption of vitamin B6 may be lower in patients with malabsorption or following gastric resection. The main storage site for vitamin B6 is the liver with lesser amounts being stored in the muscle and brain.

The active form of vitamin B6, pyridoxal 5' phosphate (P5P) is involved as a cofactor in more than 140 distinct enzymatic reactions in the body. The enzymes involved include: oxidoreductases, lyases, transferases, hydrolases and isomerases. These P5P dependent enzymes are involved in many physiological processes such as amino acid metabolism, fatty acid metabolism, degradation of glycogen, and hemoglobin formation. In addition, P5P plays an important protective role as an antioxidant [1].

All forms of Vitamin B6 are converted to the active form pyridoxal -5-phosphate (P5P) by pyridoxal kinase. This enzyme is expressed in many tissues such as brain, red blood cells and liver; however the majority of plasma P5P is formed in the liver. Pyridoxal 5' phosphate in the circulation is strongly associated with albumin and hemoglobin. The unbound form will be

dephosphorylated by phosphatases to pyridoxal, which will be further oxidized by the aldehyde oxidase to the ultimate degradation product 4-Pyridoxic acid (4-PA). The metabolism of vitamin B6 in laboratory animals like rats is similar to that reported in humans [4].

Several drugs have been identified as inhibitors of pyridoxal kinase and thereby vitamin B6 metabolism. According to the mechanism of inhibition, these drugs are classified into three groups:

1. The first group of drugs, which includes isoniazid, dopamine, cycloserine and thiamphenicol glycinate, forms covalent complexes with pyridoxal or P5P, via Schiff's base formation. These complexes will directly inhibit pyridoxal kinase.
2. The second group of drugs includes theophylline and progabide, which are true inhibitors of pyridoxal kinase that compete with the known substrates such as pyridoxal or pyridoxamine, however, without any complex formation.
3. The third group of drugs includes levodopa, D-penicillamine, and muzolimine, also forms a complex with either pyridoxal or P5P, however, these complexes have no inhibitory effect on pyridoxal kinase, but they rather prevent the substrate from interacting with pyridoxal kinase [5].

Vitamin B6 has been used for several non-nutritional indications as well. These indications include, but are not limited to, depression, impaired glucose tolerance and diabetes mellitus, morning sickness, and carpal tunnel syndrome [6]. The therapeutic doses vary with the indication, and typically involve high doses (50 -200 mg) of vitamin B6.

Several indicators of vitamin B6 status have been developed and utilized clinically. These indicators include direct measures such as plasma or erythrocyte vitamin concentration, or urinary 4-pyridoxic acid; and functional measures such as stimulation or activation of

erythrocyte aspartate aminotransferase (α-EAST) and alanine aminotransferase (α-EALT) by pyridoxal 5` phosphate. Less frequently used tests are the methionine load test, where an increase in methionine metabolites such as homocysteine and cystathionine indicate the vitamin B6 status. In another test, the tryptophan load test, the urinary xanthurenic acid (XA) is measured. The following table summarizes all of these tests and their respective reference values [7].

Table 1. Parameters for assessment of vitamin B6 status and reference values

Parameter		Reference value
Direct	Plasma pyridoxal 5` phosphate (P5P)	>30 nmol/L
	Total vitamin B6	>40 nmol/L
	Urinary 4-pyridoxic acid excretion (4-PA)	>3µmol/day
	Urinary total vitamin B6	>0.5 µmol/day
Indirect	α-EAST	<1.8 (80%)
	α-EALT	<1.25 (25%)
	XA excretion (2 g L-Tryptophan)	<65µmol/day
	Cystathionine excretion (3 g L-methionine)	<350 µmol/day

1.2 VITAMIN B6 DEFICIENCY

Vitamin B6 deficiency is not uncommon and has been reported in different pathological conditions such as cirrhosis, malabsorption, uremia, hyperthyroidism and congestive heart failure. Morris and colleagues in a large epidemiological study evaluated the prevalence of vitamin B6 deficiency in the U.S population. The results suggested that about 11% of those who took a daily supplement, and 25 % of those who did not take any supplement have a plasma P5P level of less than 20 nmol /L. The study concluded that the recommended daily allowance of vitamin B6 of 2 mg/day may be inadequate in certain vulnerable patient populations [8]. However, biochemical deficiency does not always lead to clinical consequences where symptomatic manifestations occur. Actually, clinical deficiency is rare, and once detected can be easily corrected by supplementation. In many cases, vitamin B6 deficiency is overlooked especially in the absence of clinical signs and symptoms. Symptoms of vitamin B6 deficiency due to either disease state or concomitant medications have been observed and they usually manifest as neurological problems [6]. The most common manifestations of vitamin B6 deficiency include hematological, immunological, cognitive, neurological and cardiovascular abnormalities. Vitamin B6 deficiency has been associated with impaired immune function that is characterized by decreased lymphocytes and interleukin-2 (IL-2) production. Some studies have suggested a significant inverse correlation between serum pyridoxal 5` phosphate and some types of cancer such as colorectal, pancreatic, gastric, oral and pharyngeal, lung, prostate and breast. The role of vitamin B6 in neurocognitive functions is well established, especially in seizures and different neuropathies. Finally, vitamin B6 deficiency has also been associated with some cardiovascular abnormalities like atherosclerosis and myocardial infarction [7].

Vitamin B6 deficiency has been reported in different solid organ transplant patients. Vitamin B6 deficiency has been reported in almost all intestinal and multivisceral transplant patients. This was the first report that highlights such deficiency in this population. In a 1- year prospective study of intestinal and multivisceral transplant recipients (isolated intestine (n=19) combined liver intestine (n=3), multivisceral (n=8)), P5P deficiency was observed in 96% of the recipients with a median onset of 30 days after transplantation. Of this group 41 % were receiving parenteral nutrition, 41% were receiving enteral feeding, and 18% had complete nutritional autonomy. The overall cumulative risk was 24% at 25 days, 59% at 30 days, 79% at 45 days and 90% at 90 days. In these patients, baseline P5P levels ranged from 9.7 – 301.5 nmol/L [9].

Vitamin B6 deficiency has been reported in as much as 60 % of the liver transplant patients. Bosy-Westphal and coworkers observed lower P5P levels in a subgroup of orthotopic liver transplant patients (OLT) (n=54), compared to a control group (n=25). The P5P plasma levels in the control group were 64 (±54) nmol/L, with 12% of them with less than 20 nmol/L, while the P5P levels in the OLT group were 30 (±49) nmol/L, with 61% of them with less than 20 nmol/L. The levels were lower in OLT with normal serum creatinine, when compared with the OLT patients with elevated creatinine. The P5P levels were 17 (±13) nmol/L, with 67% less than 20 nmol/L and 58 (±78) nmol/L, with 56% less than 20 nmol/L, respectively. Furthermore, P5P levels were inversely correlated with alkaline phosphatase levels in serum after OLT ($r=-0.32$, $P<0.05$). As P5P is almost 90% bound to plasma proteins, less albumin in liver transplant patients might lead to increased free fraction and correspondingly increased degradation of the free P5P by alkaline phosphatase [10].

Vitamin B6 deficiency has also been reported in more than 50% of kidney transplant patients. In 1983, Lacour *et al.* reported that in 116 non-uremic kidney transplant patients the mean plasma P5P was 33.8±3.5 nmol/L. In 65% of these patients the levels were < 20 nmol/L. The immunosuppressant regimen used in these patients was azathioprine and corticosteroid [13]. Furthermore, Plessis and coworkers compared P5P levels in two groups of renal transplant patients (less than 28 months post-transplant and more than 28 months post-transplant). The vitamin B6 levels were below normal in 56% in the first group and 59% in the second group [14].

Vitamin B6 deficiency has been reported in more than 20% of heart transplant patients. In one hundred and sixty patients who underwent orthtopic heart transplant the P5P level was measured along with other vitamins and biomarkers. The mean P5P concentration was ranged from 66 to 72 nmol/L. A deficiency was seen in 21% of recipients with cardiovascular complication and 9% in recipients without complications. Furthermore, survival in those with vitamin B6 deficiency was significantly lower than in those without a deficiency [15]. Similar results of lower P5P levels were reported in another group of 189 cardiac transplant patients when compared to healthy controls (40±25 vs. 84±77 nmol/L, respectively) [16].

The level of alkaline phosphatase has been correlated with P5P levels in plasma (normal level 20- 140 IU/L). The levels of P5P are markedly increased (21- fold) in clinical cases of hypophosphatemia (low alkaline phosphatase levels) [17]. On the contrary, elevated levels of alkaline phosphatase (like in cases of inflammation, trauma etc.) will lead to lower P5P levels.

Increased levels of alkaline phosphatase, which plays an important role in degradation of P5P, have been reported in different transplant patients. Hranjec and colleagues reported a case of high alkaline phosphatase in a liver transplant patient and attributed the increase in alkaline

phosphatase to Epstein - Barr virus and the immunosuppressant regimen used that included tacrolimus, mycophenolate mofetil and corticosteroid [18]. Similarly, three renal transplant patients have been reported to have elevated alkaline phosphatase levels for idiopathic reasons. The immunosuppressive regimen in all the three cases included tacrolimus [19]. These observations led to the suggestion of a potential role of tacrolimus as a cause of the observed P5P deficiency.

The success of solid organ transplantation is strongly related to advances in immunosuppressive therapy. The major issues with early transplantation were high failure rates and frequent acute rejections. However with new immunosuppressive agents like cyclosporine and tacrolimus, the rejection rates currently are much lower. After a few decades of use, the concentration has been shifted from just preventing the acute rejection to balancing that with less adverse effects. One of many adverse effects observed after organ transplantation is the poor nutritional status of the transplant patients. This is important as many of the patients undergoing transplantation are nutritionally vulnerable [15]. For example, the transplant candidates especially for small bowel transplantation will be usually receiving parenteral nutrition (PN) as their primary nutritional support.

The immunosuppressive medications used in different organ transplant populations reporting decreased P5P levels are varied. Tacrolimus was the primary immunosuppressant used in all the small bowel transplant patients. The immunosuppressive therapy regimens used in liver transplantation included cyclosporine, tacrolimus, azathioprine and mycophenolate mofetil. In Kidney transplantation, the immunosuppressant regimen was cyclosporine, azathioprine and corticosteroids. Finally, the heart transplant patients' immunosuppressive regimen included azathioprine, cyclosporine and prednisone

The proposed mechanisms for vitamin B6 deficiency in transplant populations include the increased metabolic demand that will exhaust the vitamin B6 sources, inadequate intake, abnormal metabolism (decreased formation), or drug interaction [9]. Finally, a metabolic abnormality is most likely to occur, either decreased formation (inhibition of pyridoxal kinase) or increased degradation (increased alkaline phosphatase). This last mechanism has never been tested in this patient population.

1.3 SMALL BOWEL TRANSPLANTATION

1.3.1 Small bowel transplantation

Given the highest incidence of vitamin B6 deficiency in small bowel transplant patient, we will now focus on small bowel transplant patients. The small bowel known as small intestine is the major site of absorption of drugs and nutrients. The small bowel is about six meters in length that extends from the stomach to the colon and is composed of three distinct regions: duodenum, jejunum and the ileum [20]. The structure of these segments varies significantly, along with a change in the pH from being slightly acidic in the beginning to almost neutral towards the end. In addition, the stomach contents will be mixed with bile and pancreatic enzymes in the upper part of the small bowel [21]. Drug and nutrient absorption mainly takes place throughout the intestine via different mechanisms that range from passive to active pathways. In addition, drugs and nutrients can concurrently be metabolized and or transported (uptake or efflux). Small bowel transplantation is considered the only therapeutic option for patients with intestinal failure [22].

Intestinal failure is a condition where the absorptive capacity of the intestine is seriously compromised. This can be attributed to many factors that can range from congenital defect to disease induced malfunction or injury [23]. One of the common diseases that can precipitate such a condition is chronic irritable syndrome, known as Crohn`s disease. Less common causes may include trauma, short gut syndrome, thrombosis and intestinal obstruction [24] Most of the patients with intestinal failure become dependent on parenteral nutrition (PN); however, the only ultimate therapeutic option for intestinal failure is small bowel transplantation [25]. The type of

small bowel transplantation performed varies according to patient's requirements and can be a standalone procedure or combined with other organs such as liver [26]. While intestinal failure is the main indication for small bowel transplantation, it has been performed with other organs for extended list of indications that include intestinal failure with cirrhosis, complete portal mesenteric thrombosis, slow- growing central abdominal tumors, intestinal pseudo obstruction, and frozen abdomen [27]. Transplantation may in essence include small bowel alone, liver-small intestine, the whole gastro-enteric system with liver and pancreas, and the new emerging procedure that includes stomach-duodenum-pancreas and intestine without the liver [28].

The function of the transplanted organ in terms of absorption, immunity and motility is of importance post transplantation. Absorption capacity is usually inferred from achieving the nutritional autonomy, normal height/age ratio, and body mass index. The immunity is monitored by intraepithelial lymphocyte as the first line of host immunity, while the motility is expected to recover slowly with re-innervation [29]. The immunological complications after intestinal transplantation is a major challenge and several trials have suggested the use anti-tumor necrosis factor α (anti-TNF-α) to prevent or treat rejection [30, 31]. Due to the complexity of the surgery and the high immunogenicity of the transplanted organs, there is a higher incidence of severe rejection and re-transplantation [32]. In addition, the donor specific antibody is also a unique challenge in small bowel transplants that may require the use of additional therapies against anti-donor antibodies [33]. Several immune monitoring techniques are utilized similar to other organ transplantation; however a few additional monitoring techniques such as flow cytometric mixed leukocyte responses, measurement of fecal calprotectin and single-nucleotide polymorphisms associated with inflammatory bowel diseases are also utilized [34].

1.3.2 Medication use after small bowel transplantation

Many small bowel transplant recipients like other solid organ transplant patients receive multiple medications as early as a few days prior to the surgery and will continue on most of these medications for the rest of their lives. The primary medication used in transplant patients is usually tacrolimus, which is the main immunosuppressant that is targeted to a therapeutic concentration range of 10-15 ng/ml.

In addition to the immunosuppressant tacrolimus, many other medications are commonly prescribed to small bowel transplant patients. The following table summarizes different drugs that are commonly prescribed in this patient population.

Table 2. Medications commonly prescribed to small bowel transplant patients

Immunosuppressant	*Pneumocystis Carnii* Prophylaxis	Cytomegalovirus Prophylaxis	Supplements	Anti-fungal Prophylaxis	Others
Tacrolimus	Sulfamethoxazole - Trimethoprim	Adefovir	Vitamin D3	Itraconazole	psychotropics
Cyclosporine	Dapsone	Acyclovir	Vitamin B12	Fluconazole	Anti-hypertensive
Hydrocortisone	Atovaquone	Valganciclovir	Folic Acid	Voriconazole	Antibiotics
Prednisone			Biotin	Nystatin	Antihistamines
Azathioprine			Vitamin B2		Pain Med
Sirolimus			Niacinamide		PPI's
			Vitamin B6		
			Vitamin A		
			Vitamin B1		

1.4 TACROLIMUS

Since Tacrolimus is used in all small bowel transplant patients, we will now focus on a discussion of this medication. Tacrolimus is the immunosuppressant of choice in all solid organ transplantations like liver, kidney and small bowel. Known as FK-506, it is a 23-memebered macrolide lactone, which is isolated from the fermentation broth of *Streptomyces tskubaensis*. Tacrolimus inhibits lymphocyte proliferation by binding to FK-binding proteins (immunophilins), which in turn inhibits the activity of calcineurin (a serine threonine phosphatase). Calcineurin will be unable to dephosphorylate the nuclear factor of activated T cells ($NFAT_c$), resulting in suppression of IL-2 transcription.

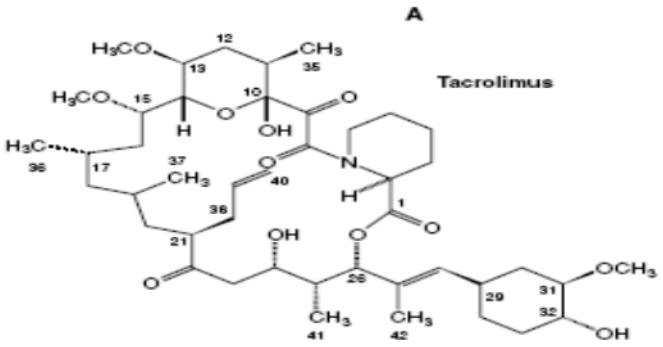

Figure 1. Structure of Tacrolimus

Tacrolimus has a narrow therapeutic index and requires routine monitoring of its concentration in whole blood to decrease the probability of rejection and minimize toxicity. The

desired trough blood concentration of tacrolimus depends on the organ transplanted and other co-administered immunosuppressive drugs and is usually maintained between 8-15 ng/ml. The main adverse effects associated with tacrolimus are neurotoxicity, nephrotoxicity, and electrolyte disturbances like hypomagnesaemia and hyperkalemia [35].

Tacrolimus pharmacokinetics and drug interactions have been extensively studied. Briefly, the bioavailability from orally administered tacrolimus ranges from 5- 67%, with an average of 29% in liver, kidney, or intestinal transplant patients. This low bioavailability has been attributed to gut metabolism (CYP3A) and gut efflux (p-glycoprotein) [36]. Tacrolimus distributes between blood cells and plasma with a mean blood to plasma ratio 15:1. Clinically, whole blood tacrolimus concentrations are usually measured instead of plasma concentrations. In blood, tacrolimus is associated with erythrocytes, while in plasma it is associated with $alpha_1$-acid glycoprotein (67-91% bound) and to albumin (69% bound) [36]. Finally, tacrolimus is mainly cleared from the body by CYP3A4 enzymes into at least nine different metabolites that are primarily secreted in the bile. Tacrolimus is subjected to several drug interactions, and many of these interactions are either CYP3A based inhibition (azoles), CYP3A based induction (rifampicin), or P-glycoprotein transporter based interactions. Other types of drug interactions that are not CYP3A or P-glycoprotein based have not yet been investigated [35].

There were no reports of interaction in the literature between tacrolimus and vitamin B6. The documented vitamin B6 deficiencies in most patients on tacrolimus give us a basis for our hypothesis of a potential effect of tacrolimus on vitamin B6. Tacrolimus may lead to vitamin B6 deficiency by either decreasing the formation of P5P, by inhibiting the enzyme pyridoxal kinase and/or increasing the degradation of P5P by elevating the levels of alkaline phosphatase. and consequently their physiological functions.

1.5 VITAMINS STATUS FOLLOWING TRANSPLANTATION

1.5.1 Introduction

Vitamins are required by the body to carry out several vital functions. They are usually needed in small quantities. There have been many reported serious complications that were due to specific vitamin deficiencies. These complications can range from anemias, neuropathy, and osteoporosis to well-defined disease states like scurvy and beri-beri. Alterations in vitamin status after solid organ transplantation have also been reported in the literature. These have been primarily deficiencies that include both fat soluble vitamins (A, D, E, and K), as well as water soluble vitamins (B1, B2, B6, B3, and B12). The extent and severity of deficiency varies considerably depending on the type of organ transplanted and the perioperative nutritional support used. Often, vitamin deficiencies are associated with organ dysfunction and are normally corrected once a new allograft is received. The purpose of this review is to evaluate the vitamin status following solid organ transplantation.

Altered fat soluble vitamins status has been reported after kidney and liver transplantations. Vitamins status may be affected after transplantation due to inadequate intake or supplementation, disturbed activation by different organ systems, or by immunosuppressive medications used. For Example, renal transplant recipients have a higher prevalence of vitamin D deficiency as the kidney is the site of activation of vitamin D into 1:25-hydroxyvitamin D (37). The prevalence of deficiency and insufficiency in this population is approximately 70-80% [38]. The deficiency has been associated with secondary hyperthyroidism and hypocalcaemia [39]. Although, the prevalence of deficiency is high regardless of transplant status (recent or

long-term), it has been reported to occur in most of the African American kidney recipients [40]. Interestingly, a recent study reported severe vitamin D deficiency among heart and liver transplant recipients despite vitamin supplementation [41]. Likewise, the plasma concentrations of vitamin A, a fat-soluble vitamin, which is predominantly stored and metabolized in liver, was lower post liver transplant patients [42]. Studies reported that both vitamin A and vitamin E levels are lower in patients with cirrhosis before transplantation [43, 44]. Increased levels of both fat soluble vitamins (vitamin A and vitamin E) have been reported in adult cystic fibrosis patients after lung transplantation [45]. The exact requirements of supplementation for fat soluble vitamins may differ depending on the type of organ transplanted. While vitamin K is important for the biosynthesis of procoagulant serum protein factors in the liver [46], vitamin E may have beneficial effects in heart transplantation.

Similarly, deficiency of water soluble vitamins has been reported in patients undergoing different organ transplantation. Vitamin B6 deficiency was significant following intestinal transplantation and to lesser extent in kidney, liver and cardiac transplantation [9, 12]. Similarly, acute folic acid deficiency was observed after bone marrow transplantation [47]. However, there are reports of normal levels of B-group vitamins after renal transplantation [48].

Table 3. Summary of vitamin deficiencies following solid organ transplantation

Vitamin	Organ
Vitamin A	liver
Vitamin D	Kidney, liver
Vitamin E	Liver, Lung
Vitamin K	liver
Vitamin B6	Small bowel, heart, liver, kidney
Vitamin B12	heart
Thiamine	Kidney, liver

1.5.2 Fat soluble vitamins

The fat soluble vitamins include vitamin A, D, K, and E. Vitamin D was among the most studied vitamin, especially in kidney transplant patients. Vitamin concentrations in various solid organ transplantations will be summarized below.

Vitamin A

Vitamin A and its metabolites are important for cellular growth and play an important role in differentiation of various epithelial and immune cells. It is very important for growth and vision as well as for its antioxidant protective role. The naturally occurring ester form of vitamin A is usually hydrolyzed to retinol that is taken up by the intestinal mucosa, re-esterified back,

coupled with chylomicrons, and finally released via the thoracic duct into the circulation. The remnants of the carrier – containing most of the ester form- are taken by the liver and stored after hydrolysis as retinol. The serum level of vitamin A is maintained through homeostatic regulation from the liver stores. Retinol is bound to retinol-binding protein (RBP) before being released and further coupled to transthyretin (TTR) to minimize renal elimination. In the cells, retinol is bound to cellular RBP, oxidized to retinoic acid, or re-esterified again. The ester form is stored in different tissues in addition to liver, where 50-80% of the body store is maintained [49]. Cytochrome P450 26 hydroxylation as well as glucuronidation are involved in this process.

Vitamin A status following transplantation

Vitamin A deficiency, defined as plasma retinol levels of less than 1μM, has been reported in patients with end-stage liver disease awaiting transplantation. Both plasma retinol (normal level 1.6 – 2.3 μM) and serum retinol binding protein RBP (normal level 1.4 – 2.9 μM) were lower in pre liver transplant patients (100 and 95% of the patients, respectively), than normal subjects. Similarly, 82% (n=77) of pretransplant patients with primary sclerosing cholangitis had serum vitamin A levels that were below the normal range [50]. Ukleja and co-workers reported significantly lower levels of serum and hepatic vitamin A levels as well as the RBP in fifty patients with cirrhosis compared to matching control subjects [44]. Dark adaptation that resulted from vitamin A deficiency in patients waiting for liver transplantation was improved by intramuscular vitamin A treatment [51].

Following liver transplantation, a beneficial effect on restoring the nutritional status of patient with cystic fibrosis was noted. Both vitamin A and vitamin E levels increased ($P<0.05$) subsequent to transplantation [52]. In addition, a case report of a patient with severe visual field restriction that failed vitamin A supplementation showed improvement following liver

transplantation [53]. However, a case of night blindness secondary to vitamin A deficiency has been reported after transplantation also. This patient likely developed a biliary stricture as a complication of the transplantation procedure [54]. Serum vitamin A and retinol binding protein were significantly reduced in the kidney transplant patients during the immediate post-transplant period, but consistently increased several years after transplantation [55].

Vitamin D

Vitamin D that exists in two different forms - vitamin D2 (known as ergocalciferol) and Vitamin D3 (known as cholecalciferol), is best known for its beneficial effects on bone health. It is known to be involved in the intestinal absorption of minerals like calcium, iron, magnesium, and phosphate. Vitamin D is normally absorbed from the intestine into the circulation where it binds to vitamin D-binding protein (DBP). The two forms of vitamin D (D2 and D3) need successive hydroxylation in the liver and kidney to form active 1, 25-dihydroxyvitamin D. It is transported to liver, where it is hydroxylated to 25-hydroxy vitamin D, by microsomal CYP450 (CYP2R1) and/or mitochondrial CYP450 (CYP27A1). In kidneys, 25-hydroxyvitamin D3-1α-hydroxylase (CYP27B) will ultimately add another hydroxyl group at position 1 to convert 25-hydroxy vitamin D to the dihydroxy active form. The normal vitamin D status, usually measured by 25-hydroxy vitamin D levels, is considered to be at least than 50 nmol/L or 20 ng/ml. New assays are able to measure the 1, 25 di-hydroxy vitamin D metabolite and the normal range is between 16 and 60 pg/ml [46, 56].

Vitamin D status following transplantation

Vitamin D deficiency has been reported in transplant candidates with congestive heart failure, end-stage pulmonary disease, liver failure and most commonly chronic kidney diseases

[44]. Transplantation unfortunately will not restore vitamin D status regardless of the transplanted organ. Estimates of prevalence of vitamin D deficiency following transplantation were variable according to patient populations, type of transplanted organ, and are also dependent on assay used to measure 25-hydroxy metabolite. The vitamin D insufficiency is not uncommon and it is estimated that 26-33% of transplant patients developed severe deficiency [41]. Low vitamin D levels have been suggested to be utilized in kidney transplant patients as a predictor of worsening of graft function and increasing proteinuria [37]. About 40-50% of patients were vitamin D deficient post-renal transplantation and almost 90% of patients were vitamin D deficient after liver and/or heart transplantation. The etiology of vitamin D deficiency varies and can range from malnutrition, lack of sun exposure, steroid therapy, alterations in metabolism by the liver [38, 39, and 41]

Vitamin E

Vitamin E, known as α-tocopherol, is poorly absorbed through intestinal tract and transported via lipoproteins and chylomicrons to liver and other tissues. Vitamin E is metabolized by cytochrome P450's then conjugated and excreted in urine or bile. Hepatic CYP4F2 is primarily involved in metabolizing vitamin E; in addition CYP3A may also be involved. Vitamin E deficiency is defined as α-tocopherol plasma concentration of less than 12 μmol/L [46].

Vitamin E status following transplantation

Vitamin E deficiency had been reported in patients with alcohol-related liver disease, viral hepatitis, and hepatocellular carcinoma [44]. Forty three percent of pretransplant patients with primary sclerosing cholangitis had vitamin E levels below normal [50]. The levels of

vitamin E were increased significantly ($p<0.05$) in cystic fibrosis patients following liver transplantation when compared with non-transplanted patients [52]. Similar findings with lung transplant recipients have also been reported [45].

Vitamin K

Vitamin K, found mainly in green plants as a known phytonadione, is absorbed from the intestine via the lymphatic system. Tissue carboxylation as well as hepatic reductases has a role in conversion of vitamin K to the active epoxide form. The deficiency is rare; however it has serious consequences .The normal plasma concentration is estimated to be 1.0 nmol/L (0.45 ng/ml) [46]

Vitamin K has a crucial role in the biosynthesis of procoagulant serum protein factors in the liver [57]. Chronic liver diseases usually require vitamin K supplementation to restore hemostatic abnormalities [58]. Few studies have evaluated vitamin K status prior to transplantation as the deficiency can occur due to many different reasons. These reasons include: drug antagonism, liver dysfunction, inadequate intake and/or malabsorption. About 30% of children patients (8/26) pre-bone marrow transplantation had vitamin K deficiency [59].

Vitamin K status following transplantation

Vitamin K deficiency after transplantation was reported in a series of case reports after kidney and combined kidney-pancreas transplantation. Four patients from a total of 146 transplant patients developed vitamin K deficiency induced coagulopathy and bleeding 1-week after transplantation [60]. Hemostatic abnormalities associated with liver transplantation are rather inherently related to different surgical phase of the transplantation and no data is available about vitamin K status post liver transplantation [58].

1.5.3 Water soluble vitamins

Water soluble vitamins are less frequently evaluated and reported in the transplant literature. Only vitamin B1, B3, B6, and B12 levels have been reported in different solid organ transplant patients. Vitamins status of patients after solid organ transplantation will be summarized separately.

Vitamin B6

Vitamin B6, known as pyridoxine, is composed of three naturally occurring distinct chemical forms as free pyridoxamine, phosphorylated pyridoxal and pyridoxal. Pyridoxamine and the other forms will be absorbed passively in the jejunum. The non-phosphorylated forms will cross into the circulation. Pyridoxal binds to albumin and erythrocytes where it will bind to hemoglobin. The majority of the absorbed vitamin B6 in the unphosphorylated form is transported to liver by simple diffusion where metabolism by pyridoxal kinase into the active form pyridoxal-5 phosphate (P5P) takes place. The active form P5P is either released into plasma or is bound to Apo-enzymes. The P5P undergoes hydrolysis by non-specific alkaline phosphatase. Most excess pyridoxal in tissues will be oxidized to urinary 4-pyridoxic acid by liver and kidney [61]. The normal level of P5P is between 30-80 nmol/L and the total body storage is estimated to be about 167 mg.

Vitamin B6 deficiency is not uncommon and has been reported in different pathological conditions in patients with alcoholism, cirrhosis, malabsorption, uremia, hyperthyroidism and congestive heart failure. Morris and colleagues in a large epidemiological study evaluated the prevalence of vitamin B6 deficiency in U.S population. The results suggested that about 11%

who took a daily supplement, and 25 % who did not take any supplement have a P5P level of less than 20 nmol /L [8].

Vitamin B6 status following transplantation

Recently, Matarese *et al*. reported a high incidence of vitamin B6 deficiency following small bowel and multivisceral transplantation ~96% [9]. Variable degrees of deficiencies have also been reported in liver transplant patients (60 %) [10], renal transplant patients (50 %) [11] and, cardiac transplant patients (20 %) [12]. Vitamin B6 deficiency occurred in about 59% of renal transplant recipients after more than 28 months post transplantation [14]. In another study, vitamin B6 levels were lower in cardiac transplant patients than controls and the deficiency was seen in about 17.9 % of the transplant patients in comparison to 2.2 % in controls [16]. However, the mechanism of this deficiency has not been investigated so far.

Vitamin B12

Vitamin B12, known as cyanocobalamin- is actively absorbed through a highly specific mechanism in the ileum that depends on intrinsic factor and its receptors. The liver and the kidneys are the richest repositories of vitamin B12, also known as cobalamin, in the body. Vitamin B12 is metabolically active as coenzyme B12 and as methylcobalamin. Three soluble B12 binding proteins (intrinsic factor, transcobalamin, and haptocorrin) are known to be involved in the uptake and transport of cobalamins in human [61]. A significant amount of vitamin B12 is secreted in the bile; however, about 70% is normally re-absorbed. The normal serum cobalamin value ranges between 200 and 250 ng/L. The deficiency usually is due to inadequate intake, gastrointestinal malabsorption, or metabolic disorders such as nitrous oxide toxicity [46]. In addition to serum cobalamin levels, serum methylmalonic acid concentrations have been utilized as an index of tissue cobalamin status [62].

Vitamin B12 status following transplantation

Gupta *et al.* studied 189 orthotropic cardiac transplant recipients and found that cobalamin deficiency was present in 4.3% of their patients and in 5.3 % of controls [16]. Vitamin B12 deficiency was not significantly different between heart transplant patients with and without cardiovascular complications and furthermore, vitamin B12 concentrations failed to predict complications or death [12]. Similarly, in renal transplant patients (n=55) there were no differences in the mean vitamin B12 levels compared to the matched control group (n=32) [48].

Vitamin B1

Vitamin B1 is a water soluble B-vitamin known as Thiamine. Best known for its neurological effect as it is involved in neurotransmitter synthesis and in the treatment of alcoholism. Tissue thiamine deficiency was suggested as a potential cause of delayed graft function after kidney transplantation, which was improved by thiamine supplementation to the donors (63). Thiamine supplementation improved signs and symptoms of a liver transplant patient who developed hemorrhagic Wernicke's encephalopathy [64]. Wernicke's encephalopathy is a metabolic disorder that is mainly caused by thiamine deficiency and frequently associated with chronic alcoholism and some forms of malnutrition or malabsorption. Few cases of this disorder were similarly reported after allogenic peripheral blood stem cell transplantation [65, 66].

Vitamin B3

Vitamin B3, known as Niacin, is obtained from the diet from in the form of tryptophan, nicotinic acid and nicotinamide. All utilized for synthesis of NAD that mediates many biochemical redox reactions. Pellagra, which results from a deficiency of niacin, can lead to

severe consequences and eventually death. Nicotinic acid is converted in the intestine into nicotinamide, which is the predominant form in the circulation. Many cellular transporters were identified to transfer both chemical forms into various cells. However, nicotineamide is also the degradation product of the pyridine nucleotides [67]. There were no reported deficiency in niacin following transplantation, however it is very likely as niacin metabolism is largely dependent on vitamin B6 as an essential co-factor. Further studies are essential in this area.

1.5.4 Conclusion

Deficiencies in various vitamins have been reported following different solid organ transplantations. The underlying causes have been attributed to organ specific function associated with that specific vitamin. Dietary intake, changes in metabolism and other biochemical processes attributed to transplantation or immunosuppression were stated as the reason for deficiencies observed without adequate evidence. Depending on the organ, transplantation requires variable levels of immune suppression that may affect the overall wellbeing and the long-term rehabilitation of the recipient. Different transplantation patients may experience variable degrees of deficiencies in different vitamins. Renal transplant patients are more likely to develop vitamin D deficiency, while liver transplants are more prone to develop vitamin A, D, E and K deficiency. The mechanisms underlying each deficiency also can be variable and unforeseen in many cases. This review draws attention to further mechanistic analysis of these deficiencies for better understanding of the nutritional status of organ transplant patients. Of all the transplant patients evaluated so far, small bowel transplantation appears to be

the major population with high incidence of deficiency in one of the water soluble vitamins, the vitamin B6.

1.6 HYPOTHESIS AND SPECIFIC AIMS

Vitamin B6 deficiency appears to be common in all transplant patients. The magnitude of deficiency appears to vary from one organ transplant group to another, with the highest incidence being reported in small bowel transplant patients. Different mechanisms have been suggested to be responsible for such an observation. The active form of vitamin B6 is pyridoxal 5` phosphate that is formed in the liver by the enzyme pyridoxal kinase. The active form is predominantly bound to albumin and red blood cells. Decreased plasma pyridoxal 5` phosphate in transplant patients may be due to inhibition of enzymes involved in its formation by concurrent drugs or elevated inflammatory markers like cytokines, that are typically increased in organ transplant patients. Decreased plasma pyridoxal 5` phosphate in transplant patients may be due to increased degradation by plasma alkaline phosphatase, decreased plasma protein binding of pyridoxal 5` phosphate due to lower concentration of albumin in transplant patients that may lead to increased free fraction and increased degradation, drugs administered, or surgery and elevated inflammatory markers that might alter the metabolism of various vitamins.

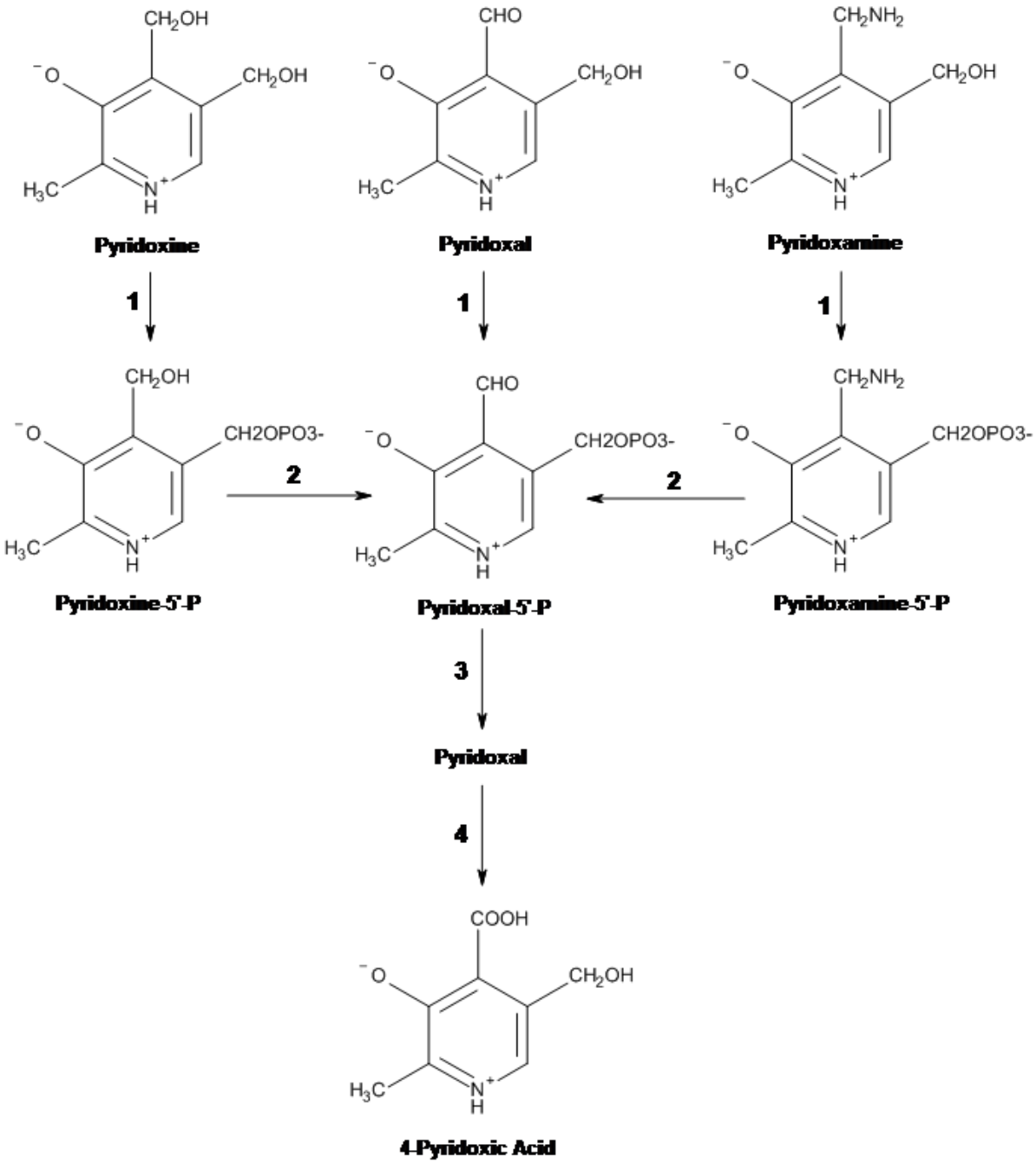

Figure 2. Biochemical processes involved in the metabolism of vitamin B6 vitamers

1:Pyridoxal kinase , 2: Oxidase, 3: alkaline phosphatase, 4: aldehyde oxidase

Hypothesis and Predictions:

Hypothesis 1: Plasma pyridoxal 5` phosphate concentrations will be decreased in transplant patients due to increased degradation mediated by elevated plasma alkaline phosphatase concentrations.

Prediction: Alkaline phosphatase levels will be increased after transplantation and there will be higher urinary excretion of the degradation product, 4-pyridoxic acid in patients urine. Urine samples will be collected from small bowel transplant patients for 12 hours period for measurement of 4-pyridoxic acid. The total amount of 4PA excreted will be compared between small bowel transplant patients and normal healthy control subjects.

Hypothesis 2: Free fraction of pyridoxal 5` phosphate will be increased due to lower concentration of albumin in patients or due to displacement by other drugs that are also bound to albumin (Tacrolimus) and this will lead to increased clearance of P5P to 4-PA

Prediction: Albumin levels will be lower after small bowel transplantation. Tacrolimus will increase free fraction of P5P in plasma. Albumin levels will be compared between small bowel transplant patients and normal healthy subjects.

Hypothesis 3: Plasma pyridoxal 5` phosphate concentration will be decreased in transplant patients due to decreased expression of pyridoxal kinase and/or direct inhibition of pyridoxal kinase involved in its formation by concurrent drugs administered.

Prediction: The medications used in transplant patients will decrease the expression and or directly inhibit the formation of P5P. A computational approach will be used to

identify the probable drug interactions of the most commonly used drugs in small bowel transplantation with pyridoxal kinase. The computer program will design the conformational structure of pyridoxal enzyme and identified the binding pockets. The chemical structure of selected medications will be screened for their virtual spatial fitness to these corresponding pockets. The docking score of each chemical structure will be recorded and compared to the docking scores of various known inhibitors that will be used to validate the correctness of the spatial configuration of the enzyme.

Hypothesis 4: Plasma P5P concentrations will be lower in transplant patients due to decreased expression or inhibition of pyridoxal kinase by increased cytokine levels.

Prediction: The following cytokines will be elevated (IL-6, IL-8, IL-10, and TNF-α) during the early post-transplant study session. Both IL-6 and TNF- α level will remain elevated at the later post-transplant session. TNF- α will significantly decrease the expression and activity of pyridoxal kinase in primary cultures of human hepatocytes.

Hypothesis 5: The process of transplantation itself will elevate inflammatory markers and will decrease the expression and activity of pyridoxal kinase.

Prediction: Transplantation of an organ that is not involved in the absorption and metabolism of vitamin B6 will alter the liver pyridoxal kinase metabolic capacity.

A composite allograft tissue transplant animal model will be employed. The liver extracts will be tested for pyridoxal kinase activity using pyridoxal as probe substrate and the expression of pyridoxal kinase in liver tissues will be evaluated using RT-PCR.

AIMS

Aim1: To a**nalyze of the causes of vitamin B6 deficiency following small bowel transplantation in patients (4-PA, Alkaline Phosphatase, Albumin) (Chapter 3).**

This study will quantitate the degradation product 4-pyridoxic acid (4-PA) in small bowel transplant patients at two different times after transplantation. The amounts of urinary 4-PA will be measured using HPLC. Plasma Alkaline phosphatase and serum albumin will be measured as part of the clinical care and the results will be collected from the patient charts. We predict increased amount of 4-PA in small bowel transplant patients in comparison to healthy subjects which is due to higher alkaline phosphatase levels.

Aim 2: To evaluate plasma cytokines levels after transplantation (Chapter 3)

Plasma cytokine levels will be measured at various time points after small bowel transplantation. The following cytokines will be measured (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INFγ, and TNF-α) at both the early and the later post-transplant study session. Each individual cytokine concentration will be compared with its concentration measured in healthy control subjects.

Aim 3: To evaluate the activity of pyridoxal kinase in human liver extract and human hepatocytes in culture in presence of tacrolimus and cytokines (Chapter 4,5).

The pyridoxal kinase activity will be measured in presence of different concentrations of tacrolimus in human liver extracts and hepatocytes with pyridoxal as the substrate and by measurement of pyridoxal 5`phosphate formed. Pyridoxal kinase mRNA

expression in hepatocytes in presence of different concentrations of tacrolimus and selected panel of cytokines will also measured with RT-PCR. A supporting *in silico* computational approach will be utilized to identify possible inhibitors.

Aim 4: To determine the effect of inflammation and transplant process on vitamin B6 metabolism (Chapter 6).

In order to obtain the highest impact of transplantation on the whole body system without involving an organ that is directly or indirectly involved in absorption or metabolism of vitamin B6, a composite allograft tissue model will be employed. Transplantation of the hind limb will be performed as follows: Iso graft (white - white), allograft (white – brown), and allograft that will receive tacrolimus (white - brown) and control groups. Different tissues and organs will be collected. The liver extracts will be tested for pyridoxal kinase activity using pyridoxal as probe substrate and the expression of pyridoxal kinase in liver tissues will be evaluated using RT-PCR.

Aim 5: To determine the effect of tacrolimus on serum protein binding (Chapter 5).

Pyridoxal 5`Phosphate binding to serum albumin will evaluated in the presence of different tacrolimus concentration. Both Pyridoxal 5`phosphate and tacrolimus bind to serum albumin, so the degree of displacement will be evaluated *in vitro* using ultrafiltration.

2.0 VITAMIN B6 METABOLITES ASSAY DVELOPMENT

Abstract

We present a simple and validated high performance liquid chromatographic method (HPLC) for measuring Vitamin B6 metabolite, 4-pyridoxic acid (4-PA) in human urine samples. Urine samples were treated with 6 % perchloric acid to precipitate any proteins. Separation of various components was achieved using (Symmetry® C_{18} 250mm x 4.6 mm I'd (5µm) column (Waters Inc.). The mobile phase used was a mixture of methanol and 35 mM sodium phosphate buffer containing 2.5 mM sodium heptane polysulfonate at pH of 3.5 with 85% O-phosphoric acid. The retention time was 8.0 min for 4-pyridoxic acid. UV detection at 302 nm for the analyte was linear in the concentration range from 0.0125 to 0.8 µM. The assay showed acceptable sensitivity and selectivity and was very reproducible. Correlation coefficient of linear fit curve for 4-pyridoxic acid obtained from data was greater than 0.99. The accuracy and precision data from back calculated calibration standards demonstrate suitability of the calibration method. Deviations from the nominal concentrations and CV values were from 1 to 16% and 3.8 to 17.5% for all concentration levels, respectively. The mean intra- and inter-day accuracy and precision for the back calculated concentrations for quality control medium (QCM) (0.25µM) and quality control high (QCH) (0.5 µM) samples were within 15% and for quality control low (QCL) (0.05 µM) samples were within 20%. The recovery from urine was (76% ± 0.2%) vs.

(104±0.8 %) from water for QCL and the recovery from urine was (88.0 ±0.8 %) vs (89.1 ±6.8 %) from water for QCH. In conclusion, this method is stable, reproducible and has practical advantages such as ease and low cost over previously published methods for analysis of 4-PA in urine.

2.1 VALIDATION OF AN HPLC-UV METHOD FOR ANALYSIS OF 4-PYRIDOXIC ACID IN HUMAN URINE

2.1.1 Introduction

Vitamin B6 is a water soluble vitamin that exists predominantly as pyridoxine. Vitamin B6 is converted by pyridoxal kinase to its active metabolite (P5P), which is involved in more than 140 biochemical reactions. Pyridoxal 5` phosphate is highly protein bound and is ultimately converted to inactive 4-pyridoxic acid that is excreted into urine.

Different analytical methods are reported in the literature for the assay of vitamin B6 metabolites in plasma and urine including spectrophotometry, fluorimetry, and HPLC. Several analytical methods utilized fluorimetric detection either directly or through chemical modification to improve sensitivity of detection. The methods included pre-column or post column derivatization [68-72]. One method utilized coulometric detection [73]. These methods were limited by the extra chemical step that either needs to occur before or after column separation. The sensitivity and accuracy of these methods were also not optimal. The aim of this

work was to develop a validated, simple and reproducible method for the estimation of the major urinary metabolite of vitamin B6 in human urine.

2.1.2 Materials and Methods

2.1.3 Reagents

4-pyridoxic acid (4-PA) was purchased from Sigma (St. Louis, MO, USA). Methanol, sodium heptane sulfonate, and orthophosphoric acid 85%, were purchased from Fluka. Sodium phosphate monobasic, sodium phosphate dibasic, animal charcoal and perchloric acid were purchased from Fischer. Reagent-grade water obtained from a Millipore Milli-Q system was used throughout the experiments. All other reagents were of analytical grade.

2.1.4 Apparatus and chromatographic conditions

The HPLC system consisted of a Waters 2695 separation module and Waters 2998 photodiode array detector. A reverse-phase column Waters Symmetry® C_{18} 250mm x 4.6 mm i.d (5µm) was used to separate the analytes, and the temperature of the column was held at 25°C. The gradient mobile phase consists of: methanol (20-80%) (phase A) and 35 mM sodium phosphate buffer (phase B) containing 2.5 mM ion-pairing agent sodium heptane polysulfonate that was adjusted to pH of 3.5 with 85% orthophosphoric acid. The buffer was sonicated, filtered and degassed prior to run. The flow-rate was 1 ml/min. Injection volume was 50µl. The detector was set at 302nm. Total run time was 10 minutes.

2.1.5 Preparation of solutions

Stock solution of 4-pyridoxic acid (1mM) was prepared by dissolving it in HPLC grade water. Stock solution was further diluted with charcoal pre-treated urine (to remove endogenous 4-pyridoxic acid) to working standard solutions with concentrations ranging from 0.0125 µM to 0.8 µM. All solutions were stored at -80◦C to simulate the storage conditions of the study samples and were found to be stable for at least 2 months. Quality control samples were prepared by separately weighing 4-PA and preparing the solutions as described earlier.

Validation of the method was performed according to FDA guidelines [74]. Peak area detected at the retention times of the analyte of interest was measured. The linearity of the assay over a range of 4-pyridoxic acid concentrations 0.0125 -0.8 µM was assessed. The recovery for each concentration was calculated against the theoretical concentration. Inter- and intra- day precision, accuracy and recovery were also calculated for all standards as well as three quality control standards (quality control low (QCL), quality control medium (QCM), quality control high (QCH)). Bench-top stability for quality control samples after 1hr and 4hrs at room temperature as well as auto sampler stability after 24 hours was also performed. Long term stability after 3 months storage and after several freeze-thaw cycles were also carried out. Finally, dilution analysis at 1:1, 1:2, and 1:4 folds were performed for quality control sample for accuracy and precision as well as recovery.

The accuracy and precision of the analytical method were evaluated by analysing quality control samples at three concentration levels (QCH 0.5 µM, QCM 0.25 µM, QCL 0.05 µM). Six

of both QCH and QCM level and twelve of QCL level were analysed daily for three runs. The calculated mean concentration was used to express accuracy (% deviation):

% deviation = (Calculated concentration value – Reference concentration)/Reference Concentration X 100

Means, standard deviations and coefficients of variation were calculated from QC values and used to estimate the intra- and inter- day precision:

% Coefficient of Variation = (Standard deviation of calculated Concentrations / Mean of calculated concentrations) X 100

2.2 RESULTS AND DISCUSSION

2.2.1 Preparation of standard and controls

Urine samples obtained from six different sources were treated with activated charcoal then analyzed according to the procedure previously described, in order to evaluate method specificity. Treatment with activated charcoal will remove all interference and to make sure there are no endogenous chemicals with retention times similar to 4-pyridoxic acid. These samples confirmed the appropriateness of using this method to prepare blank urine samples without any interference of endogenous 4-pyridoxic acid. A representative chromatogram of 4-pyridoxic acid in urine is shown in Figure 3. The retention time of 4-pyridoxic acid was 9 minutes in the run shown in figure.

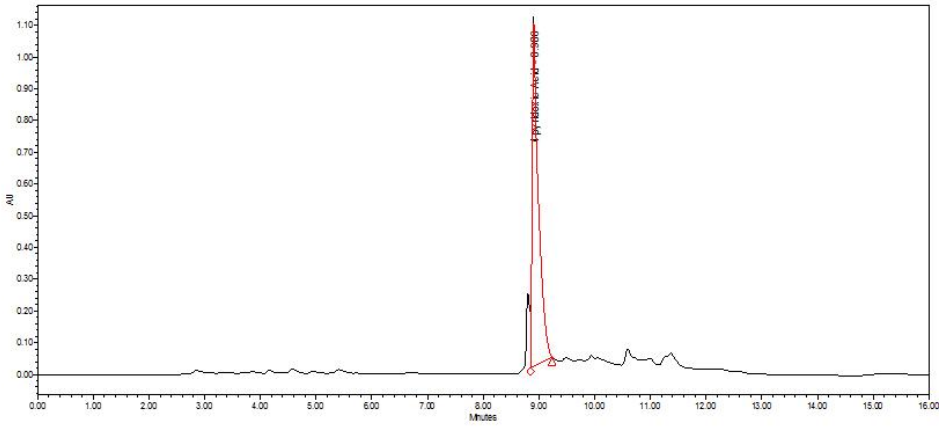

Figure 3. Chromatogram of 4-pyridoxic acid (4-PA) in urine (0.8 µM)

2.2.2 Standard Curve

Standard curve samples were analyzed in three separate runs. For each curve, the absolute peak areas of the analyte (4-pyridoxic Acid) were plotted against the nominal concentrations. Calibration curves were obtained for analyte over the concentration range of 0.0125 – 0.8 µM. The correlation coefficients (r) were greater than 0.99. The lowest standard on the calibration curve was identified as the lowest concentration where the analyte peak area is reproducible with a precision of 20% and accuracy of 80-120%. The lowest concentration should be within 20% deviation from the nominal concentration and the rest of standards should be within 15% from nominal concentration. At least four out of six non-zero standards met these acceptance criteria.

The accuracy and precision were evaluated with quality control samples of known concentrations in urine. Three quality control samples (QC) that represent the whole concentration range were prepared as follows: QCL is within 3-times the lower limit of quantification (0.05 μM), QCM is near the middle of the calibration range (0.25 μM), and QCH is near the high end of the calibration range (0.5 μM). The concentrations of calibration and quality control standards are stated in Table 4.

Table 4. Standards and quality control samples

Calibration Standard/ Quality Control (QC)	**Concentration μM**
STD 1	0.0125
STD 2	0.025
STD 3	0.05
STD 4	0.1
STD 5	0.2
STD6	0.4
STD 7	0.8
QCL	0.05
QCM	0.25
QCH	0.5

2.2.3 Linearity

Matrix-based calibration standards, in the range of 0.0125-0.8 µM, were independently prepared and analysed, on three different days for inter- and intra-day variation. For each curve, the absolute peak areas of the analyte (4-pyridoxic acid) were plotted against the nominal concentrations. Correlation coefficient of linear fit curve for 4-pyridoxic acid obtained from data was greater than 0.99 (Figure 4).

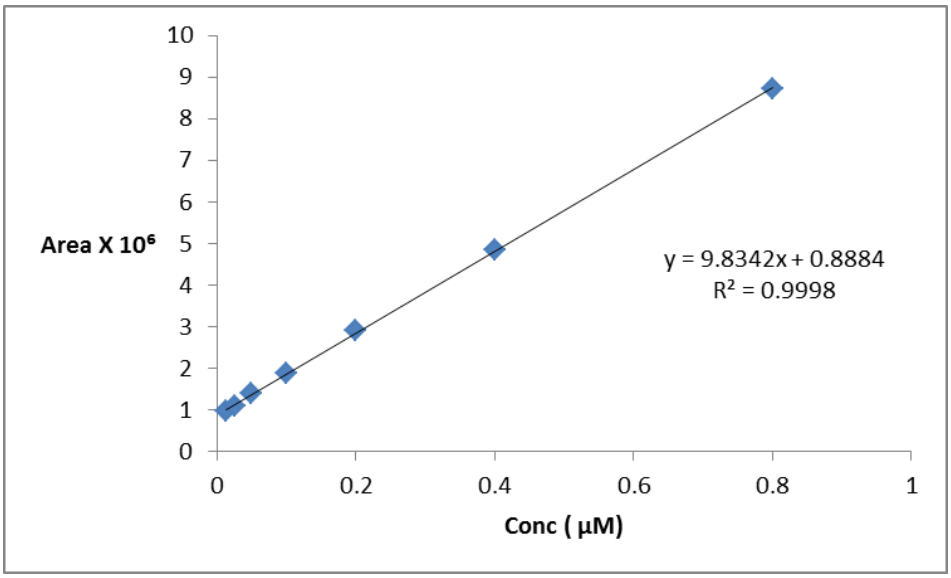

Figure 4. Calibration curve of 4-pyridoxic acid in urine

The accuracy and precision data from back calculated calibration standards demonstrate suitability of the calibration method. Deviations from the nominal concentrations and CV values were within <15% for all concentration levels, and <20% for the lowest concentration (0.0125 µM). Recovery was also > 80% at all tested concentrations. The results are shown in Table 5.

Table 5. Calibration Curve standard Concentrations accuracy, precision, and recovery.

Standard Concentration µM (n=6)	Calculated Concentration µM	SD	CV%	Deviation %	Recovery %
0.0125	0.012	0.002	17.4	-6.8	93.2
0.025	0.022	0.94	13.4	-13.1	86.9
0.05	0.050	0.003	5.1	-0.4	99.6
0.1	0.097	0.003	3.0	-2.9	97.1
0.2	0.194	0.002	1.2	-2.9	97.1
0.4	0.384	0.004	1.1	-4.0	96.0
0.8	0.765	0.012	1.5	-4.3	95.7

The accuracy and precision of the analytical method were evaluated by analysing quality control samples at three concentration levels (QCH 0.5 µM, QCM 0.25 µM, QCL 0.05 µM). Six of both QCH and QCM level and twelve of QCL level were analysed daily for three runs. The obtained values for accuracy and precision were within FDA requirements. The mean inter- and intra-day accuracy and precision for the back calculated concentrations for QCM and QCH samples were within 15% and for QCL samples were within 20%. Inter-day precision, accuracy

and recovery values are shown in table 6 while the intra-day values for day 1-3 are shown in Tables 7,8, and 9, consequently.

Table 6. Recovery, accuracy and **inter-day precision**, 4-Pyridoxic acid assay validation

Theoretical concentration (µM) (n=6)	**Calculated concentration (µM±SD)**	**Precision CV %**	**Accuracy Deviation %**	**Recovery %±SD**
0.0125	0.011±0.002	17.5	-16.1	83.7±4.3
0.025	0.024±0.002	8.3	-5.2	94.8±6.8
0.05	0.050±0.004	6.9	0.9	101.5±3.4
0.1	0.095±0.005	5.4	-5.4	83.9±14.7
0.2	0.201±0.008	4.0	0.4	94.8± 7.9
0.4	0.433±0.037	8.6	8.2	100.9±7.0
0.8	0.808±0.030	3.8	1.0	94.6±5.1
QCL*	0.042±0.002	5.2	-16.3	100.4±4.0
QCM	0.237±0.017	7.2	-4.9	108.2±9.3
QCH	0.507±0.017	3.4	1.5	101.0±3.8

***n=12**

Table 7. Recovery, accuracy and **intra-day** precision day 1, 4-Pyridoxic acid assay validation

Theoretical concentration (µM) (n=6)	**Calculated concentration (µM±SD)**	**Precision CV %**	**Accuracy Deviation %**	**Recovery %**
0.0125	0.011±0.002	18.6	-10.2	89.8
0.025	0.024±0.002	7.4	-4.3	95.7
0.05	0.046±0.005	8.9	3.8	103.8
0.1	0.097±0.002	1.6	-3.4	96.6
0.2	0.208±0.01	4.6	4.1	104.1
0.4	0.411±0.008	2.0	-17.9	102.7
0.8	0.81±0.011	1.4	1.2	101.2
QCL*	0.041±0.002	3.6	17.9	82.1
QCM	0.230±0.023	10.0	-9.4	90.6
QCH	0.522±0.023	4.4	4.5	104.5

***n=12**

Table 8. Recovery, accuracy and **intra-day** precision day 2, 4-pyridoxic acid assay validation

Theoretical concentration (μM) (n=6)	**Calculated concentration (μM±SD)**	**Precision CV %**	**Accuracy %**	**Recovery %**
0.0125	0.010±0.001	9.1	-21.7	80.0
0.025	0.023±0.002	10.6	-8.1	92.0
0.05	0.051±0.001	2.9	1.1	101.1
0.1	0.094±0.005	5.4	-5.7	94.3
0.2	0.194±0.003	1.3	-2.8	97.2
0.4	0.413±0.003	0.8	3.2	103.2
0.8	0.782±0.030	3.9	-2.3	97.7
QCL*	0.040±0.001	3.6	-20.0	80.0
QCM	0.243±0.005	2.1	-2.7	97.3
QCH	0.501±0.006	1.2	2.1	100.3

***n=12**

Table 9. Recovery, accuracy and **intra-day** precision day 3, 4-pyridoxic acid assay validation

Theoretical concentration (μM) (n=6)	**Calculated concentration (μM±SD)**	**Precision CV %**	**Accuracy %**	**Recovery %**
0.0125	0.011±0.001	11.2	-13.7	86.3
0.025	0.023±0.001	3.0	-7.1	92.9
0.05	0.045±0.001	1.4	-10.8	89.2
0.1	0.083±0.009	10.5	-16.5	83.5
0.2	0.176±0.002	1.2	-11.9	88.1
0.4	0.433±0.016	3.7	8.3	108.3
0.8	0.742±0.040	5.3	-7.2	92.8
QCL*	0.041±0.001	3.3	-18.6	81.4
QCM	0.218±0.012	5.3	-13.7	87.3
QCH	0.444±0.007	1.6	-11.2	88.8

***n=12**

2.2.4 Carry-over

Carry-over was evaluated by placing vials of blank mobile phase at different locations in the analysis set especially after the highest standard and QC. There was no carry-over evident in any of the blank reagent samples that were proven by the absence of any peaks in chromatogram corresponding to 4-pyridoxic acid.

2.2.5 Recovery

Recovery of 4-PA from urine was assessed by spiking analyte into water at the same concentrations as that of the quality control samples. Two spiked samples of each concentration (QCL and QCH) were analyzed using standard curves generated from urine based standards as described previously. Recovery after protein precipitation was compared with original concentration. Means, standard deviations and coefficients of variation were calculated. QCL recovery from urine was (76±0.15%) vs. (104±0.76 %) from water and QCH recovery from urine was (88±0.05%) vs. (89.1± 6.81%) from water. Results are shown in Table 10. Recovery calculation from urine standards and quality control samples were performed throughout the validation and was always ≥80%.

Table 10. Recovery from urine and water

Recovery								
	Concentration (µM) URINE				Concentration (µM) WATER			
	QCL		QCH		QCL		QCH	
	Conc.	Recovery %	Conc.	Recovery %	Conc.	Recovery %	Conc.	Recovery %
1	0.038	75.8	0.44	88.1	0.052	104.7	0.42	84.3
2	0.038	76	0.44	88.0	0.053	105.3	0.47	93.9
Mean	0.038	75.9	0.44	88.0	0.053	105.3	0.45	89.1
SD	0.00	0.15	0.00	0.05	0.00	0.76	0.03	6.8
CV%	0.197	0.2	0.053	0.053	0.72	0.72	7.7	7.7

2.2.6 Stability

Bench-top short-term stability after 1h and 4h at room temperature was studied to verify if 4-pyridoxic acid degraded over the course of analyses. Short-term stability was evaluated by analyzing triplicate of both QCL and QCH that was kept at room temperature before the extraction step. The CV% and deviation % as well as recovery were within the acceptable limits of validation. Results are shown in Table 11. The auto sampler stability was evaluated by leaving triplicates of both QCL and QCH samples at room temperature for 24h followed by HPLC

analysis. Both QCL and QCH CV%, Deviation % as well as recovery % were within the acceptable standard limits of validation. The results are shown in Table 12.

Long-term stability was studied in order to ensure that 4-pyridoxic acid in urine samples did not undergo degradation under the storage conditions before being analyzed. Triplicate samples of both QCL and QCH after 3 thaw-freeze cycles were analyzed to evaluate stability. The results of CV %, deviation %, and recovery % were within acceptable validation limits. Results are shown in Table 13. In addition, long-term stability was studied using triplicates of matrix-based QC's (QCL and QCH) stored at −80 ◦C for 3 months. The results for CV%, deviation % as well as recovery % were within the acceptable limits of validation. Results are shown in Table 14.

Table 11. Bench-top stability

		4-Pyridoxic Acid Standards (µM)		
	QCL-1HR	QCL-4HR	QCH-1HR	QCH-4HR
	0.05	0.04	0.49	0.49
	0.04	0.04	0.49	0.49
	0.04	0.04	0.49	0.49
Mean	0.05	0.04	0.49	0.49
SD	0.005	0.002	0.001	0.001
CV %	9.9	5.8	0.2	0.3
Deviation %	-6.4	-18.6	-2.5	-2.6
Recovery %	93.6	81.4	97.6	97.4

Table 12. Auto sampler stability

	Concentration(μM)	
Replicate	QCL	QCH
1	0.05	0.49
2	0.05	0.49
3	0.05	0.49
Target	0.05	0.50
Mean	0.05	0.49
SD	0.001	0.000
CV%	1.2	0.02
Deviation %	3.6	-3.0
Recovery %	103.6	97.0

Table 13. Long term stability

	Long Term Stability	
Replicate	**QCL (0.05 µM)**	**QCH (0.5 µM)**
1	0.05	0.44
2	0.05	0.42
3	0.05	0.44
Mean	0.05	0.44
SD	0.000	0.01
CV%	1.05	2.34
Deviation %	8.0	13.0
Recovery %	92.0	87.0

Table 14. Freeze/Thaw stability

	Freeze /Thaw	
Replicate	**QCL (0.05 µM)**	**QCH (0.5 µM)**
1	0.04	0.44
2	0.04	0.46
Mean	0.04	0.46
SD	0.00	0.01
CV%	1.04	2.09
Deviation%	-20.0	-8.0
Recovery %	80.0	91.0

The stability of the analyte (expressed as percentage recovery) at room temperature for 1 and 4 hours, in the auto sampler for 24 hours, in the storage conditions after 3 months, and after 3-thaw-freeze cycles was evaluated by comparing the back-calculated concentration to that of the nominal concentration. The results showed that 4-pyridoxic acid is stable under tested conditions without any loss. All recovery values were within acceptable range.

2.2.7 Dilution Analysis

Individual samples were prepared for the linearity of dilution analysis at a concentration of 2x HQC. Aliquots of the samples were then diluted 1:1, 1:2, and 1:4 with blank urine prior to analysis. Each dilution level was processed in triplicate and back calculated against the reference concentration. The mean accuracy and precision obtained for all dilution levels was within ≤20% and the recovery corresponding to nominal concentration was ≥ 80% according to FDA guidelines. The results are shown in Table 15.

Table 15. Dilution Analysis

	4-Pyridoxic acid (1µM)		
Replicate	1:1	1:2	1:4
1	0.41	0.26	0.13
2	0.42	0.22	0.13
3	0.43	0.20	0.14
Mean	0.42	0.23	0.13
SD	0.012	0.033	0.006
CV %	2.8	14.4	4.2
Deviation %	-16.6	-8.7	6.6
Recovery %	83.4	91.2	106.0

2.2.8 Summary of validation results

Table 16. Summary of validation results

Validation Parameter	4-Pyridoxic Acid	
Linear Range (μM)	0.0125 – 0.8	
Correlation Coefficient (mean of 3)	0.9971 ±0.0041	
Inter-day Accuracy (% Deviation)	QCL n=36 (0.05 μM)	-16.3
	QCM n=18 (0.25 μM)	-4.92
	QCH n=18 (0.5 μM)	1.47
Inter-day Precision (%CV)	QCL n=36 (0.05 μM)	5.19
	QCM n=18 (0.25 μM)	7.17
	QCH n=18 (0.5 μM)	3.35
Intra-day Accuracy (% Deviation)	QCL n=12 (0.05 μM)	
	Day1	17.9
	Day2	-9.4
	Day3	4.47
	QCM n=6 (0.25 μM)	
	Day1	-20
	Day2	-2.7
	Day3	2.1
	QCH n=6 (0.5 μM)	
	Day1	-18.6
	Day2	-13.7

	Day3	-11.2
Intra-day Precision (%CV) 0.05 µM	QCL n=12 Day1 Day2 Day3	3.6 10.0 4.4
0.25 µM	QCM n =6 Day1 Day2 Day3	 3.6 2.1 1.2
0.5 µM	QCH n=6 Day1 Day2 Day3	 3.3 5.3 1.6
Dilution Analysis Precision CV% 1µM	1:1 1:2 1:4	2.8 14.4 4.2
Dilution Analysis Accuracy Deviation % 1µM	1:1 1:2 1:4	-16.6 -8.7 6.6
Stability QCL(0.05 µM), QCH(0.5 µM)	Bench-Top stability 1Hr, 4Hr, Freeze/Thaw stability, Long Term Stability, auto-sampler after 24 Hr	Recovery ≥80%
Recovery	Acceptable recovery of ≥80% or ≤120%	From urine matrix

2.3 DISCUSSION

An analytical methodology for quantification of 4-pyridoxic acid in urine is described. Full validation according to the FDA guidelines was performed, and the limit of quantification level of 0.0125 µM was reached for 4-pyridoxic acid, with accuracy and precision levels within FDA requirements.

Previous analytical methods involved fluorescence detection in combination with pre-column or post-column derivatization. These assays utility is limited by the additional chemical modification of the analyte despite the reported enhancement of the detection potential and sensitivity. The fluorescence detection used is sensitive to pH and temperature changes. Post-column derivatization used different modifying agents such as sodium bisulfite [71, 75] and chlorite [72]. Pre-column conversion served the same purpose by adding the modifying agent before the chromatographic separation. Modifying agents such as cyanide are not routinely used by laboratories, because of toxicity and safety concerns [70]. Another method utilized similar HPLC settings as described here but used a coulometric detection. This method of measuring of different compounds by electrochemical detection is subjected to non-specific interference by endogenous compounds [73]. Other more sensitive analytical techniques were reported in literature including the use of ultra-performance liquid chromatography-tandem mass spectrometry. However, these techniques are costly and reserved for the use when detection of levels is needed in minimal volumes of samples such as in cerebrospinal fluids [76].

The method of separation utilized the polar feature of 4-pyridoxic acid as an analyte. The use of sodium polysulfonate as an ion-exchanger in the mobile phase accompanied by

adjustment of pH of the mobile phase is utilized to optimize the separation of 4-pyridoxic acid from other urinary components. The UV spectrophotometric detection was implemented at the 4-pyridoxic acid peak absorption wavelength (λmax=302 nm). The lower limit of quantification was determined as the lowest detectable concentration that maintains the accuracy and deviation within the 20% margin. The Calibration curve, quality controls were prepared as described and the validation assay was carried out.

The calibration curve in urine was linear with a satisfactory correlation of coefficient of >0.99. However, the intercept was not close to zero even after treating the urine with activated charcoal. The intercept is likely due to endogenous 4-pyridoxic acid or other endogenous chemicals. To reduce this source of interference, multiple sources of urine were pooled into one urine source and then treated with activated charcoal. The further testing for accuracy, precision and recovery was all within the acceptable range for the lower concentration of 20% margin for both accuracy and precision and of 80% for recovery. The rest of standards were similarly, within the 15% margin of acceptance for precision and accuracy and within the 80% acceptable limit for recovery.

The bench top stability, auto-sampler stability, short term stability, freeze/thaw as well as long term stability were all acceptable with ≥80% recovery. The analyte (4-pyridoxic acid) was detected in a reasonable short retention time of about 10 minutes with no evidence of carry-over. The quality control samples were utilized to evaluate the validity of the analytical method and all runs were accepted whenever the QC's run was optimal.

This HPLC-UV detection assay is achieved by the use of reversed-phase by addition of ion pairing (sodium heptanesulfonic acid). The gradient mobile phase was adjusted in terms of

optimal buffer, ion-paining agent, and methanol concentration. The peak was detected on a reasonably short time of 10 minutes that compared to other methods.

Limitation of the assay development and validation were limited to issues like interference from the matrix. The urine was treated with activated charcoal to obtain a blank standard that is free of interference. To improve the quality of work and minimize variability, repetitive measures of multiple replicates was practiced throughout the assay development and validation. The sample preparation, quality control preparation, and mobile phase preparation might lead to some variability, however following standard laboratory procedures and protocols minimize such a source of variation.

2.4 CONCLUSION

An analytical methodology for quantification of 4-pyridoxic acid in urine is described. Full validation according to the FDA guidelines was performed, and the limit of quantification level of 0.0125 µM was reached for 4-pyridoxic acid, with accuracy and precision levels within FDA requirements.

This method is suitable for the estimation of 4-pyridoxic acid in urine samples as a precursor of vitamin B6 status. It can be useful as a non-invasive technique to monitor vitamin B6 metabolites especially in patients who may experience deficiency due to high rate of degradation like in small bowel transplantation. The method was utilized to analyze the clinical urine samples from small bowel transplant patients. The analytical method is simple and the sample preparation is easy and the time of run is conveniently short. This analytical assay has an

adequate sensitivity to detect the 4-pyridoxic acid levels in urine without the need for chemical derivatization or post-column treatment like the existing fluorescent assays. This assay is easy to use with acceptable accuracy and precision and can be utilized conveniently to analyze clinical samples from different patient population.

3.0 VITAMIN B6 STATUS FOLLOWING SMALL BOWEL TRANSPLANTATION

Abstract: A deficiency in vitamin B6 was identified in small bowel transplant patients in a previous study and this study focused on investigating various mechanisms that are likely to contribute to such a deficiency. Urinary metabolite of vitamin B6 (4-pyridoxic acid) was measured using a validated HPLC method in small bowel transplant patients at two different time points after small bowel transplantation (early session within three months post transplantation and late session after more than three months post transplantation) and compared to matching healthy control subjects. Blood samples were also collected for the measurement of alkaline phosphatase, serum albumin and cytokines in these subjects. Transplant recipients had a significantly higher cumulative amount of 4-pyridoxic acid excreted in the urine when compared to control subjects. The amount of 4-PA was higher in the early post transplantation time period, compared to the later post transplantation time period. The concentrations of alkaline phosphatase in plasma were also significantly higher in transplant patients at both time periods than in control subjects. On the other hand, the serum albumin levels were lower in the transplant patients when compared to control group at both time periods as well. Certain cytokines (IL-1β, IL-2, and INF-γ) were notably elevated in the transplant patients when compared to control subjects. The study results suggested that increased degradation of vitamin B6 to 4-pyridoxic acid that was mediated by alkaline phosphatase as a probable primary cause of vitamin B6

deficiency in small bowel transplant patients. In addition, the lower concentration of serum albumin to which pyridoxal 5` phosphate binds may lead to higher free fraction of pyridoxal 5' phosphate and that correspondingly will increase the degradation of vitamin B6. This study is the first to investigate mechanistically the fate of the vitamin B6 metabolites in a transplant patient population.

3.1 INTRODUCTION

The success of solid organ transplantation is primarily due to advances in immunosuppressive therapy. The major concerns with organ transplantation in the 1980's were high graft failure rates and frequent episodes of acute rejections. However, with new agents, like cyclosporine and tacrolimus, the rejection rates are much lower and the graft and patient survival has improved significantly. Eventually, the attention has shifted from preventing acute rejection to balancing that with less adverse effects. Nutritional status of transplant patients has gained attention recently, as many of the patients undergoing transplantation are nutritionally vulnerable [77]. For example, the transplant candidates especially for small bowel transplantation will be usually receiving parenteral nutrition (PN) for their nutritional support.

Vitamin deficiency has been observed in various organ transplant patients. Vitamin B6 deficiency has been reported in several organ transplant patients, as discussed earlier (chapter 2). Almost all small bowel transplant patients develop deficiency after nearly thirty days post transplantation. Nearly, 60% of liver transplant patients, 50 % of kidney transplant patients, and about 20 % of heart transplant patients have been reported to exhibit vitamin B6 deficiency.

Studies in humans have suggested that vitamin B6 deficiency affects the immune function, and the main clinical signs of vitamin B6 deficiency are the neurological manifestations [78].

According to Matarese *et al.*, increased metabolic demand induced by steroids, immunosuppressive agents used, and allograft status may alter vitamin B6 metabolism or absorption and ultimately might lead to vitamin B6 deficiency in small bowel transplant patients [9]. Small bowel transplant has been evolving into an effective therapeutic alternative for patients with intestinal failure. However, it is also associated with relatively heavier immunosuppression and many systemic complications. These include relatively high prevalence of post-transplant neurological disorders of 30-60%. The common causes for these exacerbations were attributed mainly to immunosuppressive medications, infections and the metabolic demand. A retrospective analysis (n=54) identified neurologic issues after receiving small bowel transplantation with about two years follow-up. These manifestations were distributed between headache (50%), encephalopathy (43%), seizures (17%), CNS infections (7%), neuromuscular complications (7%), and tacrolimus neurotoxicity (14%) [79]. The nutritional status after small intestinal transplantation in a small cohort (n=46) showed an improvement and most of the patients restored their nutritional autonomy and quality of life measures after one year follow-up. However, there was no micronutrient follow-up or analysis in this study [80]. A prospective study with a five year follow-up involved 376 intestinal and multivisceral transplant recipients and reported an overall incidence of 18% of neuropsychiatric disorders that includes bipolar disorder, depression, anxiety, and peripheral neuropathy. These were significantly higher in pediatric recipients than in adults [81]. The improvement in nutritional status after intestinal transplantation in children (n=11) measured by weight Z-score and some biochemical parameters 1 year post small bowel transplantation was rather global. The study did not address specific

micronutrient status and relevant manifestations [82]. The intestinal absorption rate in children after small bowel transplantation (n=24) was shown to be suboptimal. However, this study relied on the ratio of ingested and absorbed energy to resting energy expenditure but specific micronutrient status was not addressed [83].

The immunosuppressive management following intestinal transplantation has been changing with the ultimate goal of reducing the incidence of acute rejection, which is the most prominent risk factor. Traditionally, the regimens include tacrolimus, rapamycin and steroids as well as different induction strategies with almtuzumab, daclizumab, rituximab and thymoglobulin. These different strategies led to different rates of acute rejection, survival and infections [84, 85]. The nutritional and intestinal effects of various immunosuppressive agents were addressed with several different agents. A significant decrease in intestinal nutrient absorption with agents like tacrolimus, rapamycin, as well as mycophenolic acid has been reported in rats. The mechanisms of those effects were not clearly elucidated and a metabolic effect such as impaired ATP-production was suggested [86, 87]. The intestinal dysfunction due to short term use of immunosuppressive drugs (tacrolimus, cyclosporine, mycophenolate mofetil, sirolimus and azathioprine) using *in vitro* rat jejunum model were controversial and suggested the disturbed resorption of glucose that might lead to malabsorptive diarrhea [88] or no effect on glucose absorption, chloride secretion or barrier function [89, 90].

The effect of tacrolimus on small bowel function is thus far not documented in small bowel transplant recipients. Immunosuppression with tacrolimus or sirolimus reduced cellular and molecular inflammatory events and subsequent dysmotility in the intestinal transplant graft in rats [91]. Tacrolimus had no documented effect on vitamin B6 metabolism in rats. However, a benefit of tacrolimus was evident in heart transplant recipient with elevated levels of

homocysteine, which is related to vitamin B6 [92]. In contrast, the effect of tacrolimus on homocysteine in cultured proximal tubule epithelial cells *in vitro* was not observed [93]. In addition, tacrolimus inhibited the vitamin B6 dependent tryptophan degradation in human peripheral blood mononuclear cells *in vitro* [94]. Tacrolimus is considered the main immunosuppressant used in small bowel transplantation, however with several adverse events that include neurotoxicity. A case of tacrolimus associated ischemic optic neuropathy and posterior reversible encephalopathy syndrome after small bowel transplantation has been reported, but the mechanism of toxicity was not further investigated [95].

Cytokines are among the markers that are regularly measured in various solid organ transplantations. Cytokines are of special interest in small bowel transplantation as the activation of intestinal immune system cells is much higher than that of analogous peripheral cells. Interleukine-6 (IL-6) and tissue necrosis factor-α (TNF-α) are among the usually monitored markers. (96) Interleukin-6, among other inflammatory markers in swine model small bowel transplantation showed a transient elevation following the surgery that returned to baseline in few days [97]. However, the chronic allograft rejection showed an elevated IL-6, TNF-α, and IL-10 after 60 -90 days following small intestine transplantation in rats [98]. Although, there is no association reported to date between inflammatory markers and vitamin B6 metabolism, vitamin B6 supplementation has been shown to improve pro-inflammatory responses in patients with rheumatoid arthritis. Plasma IL-6 and TNF-α levels were significantly decreased after 12 weeks of vitamin B6 supplementation when compared to control group [99].

An early hypogammagloulinemia was reported following small bowel transplantation (n=34) and about 59% of small bowel transplant patients developed this deficiency during the early post-transplant period with a mean of 10 days [100]. Serum Albumin following small

bowel transplantation also serves as a potential predictor for recovery. Serum albumin level during intestinal transplant rejection (n=26) was significantly lower than baseline levels [101]. Pyridoxal 5' phosphate is mainly bound to albumin that preserves it from further degradation by alkaline phosphatase and aldehyde oxidase. Lower levels of albumin can lead to increased degradation of vitamin B6.

Vitamin B6 deficiency has been reported in several organ transplant patients and almost in all small bowel transplant patients as mentioned in earlier chapters. Vitamin B6 deficiency was reported in as much as 96% in small bowel transplantation, 60 % of liver transplant patients, 50% of kidney transplant patients, and 20% of heart transplant patients. Increased levels of alkaline phosphatase, which plays an important role in degradation of P5P, had been reported under different conditions in liver transplant patients as well as few cases with kidney transplantation. Despite the fact that tacrolimus is not known to increase alkaline phosphatase levels, these case reports indicate that the levels of alkaline phosphatase should be monitored during tacrolimus treatment.

The proposed mechanisms for vitamin B6 deficiency in transplant populations include the increased metabolic demand that will exhaust the vitamin B6 sources, inadequate intake, abnormal metabolism (decreased formation), or drug interaction [9]. Chiang and coworkers reported that inflammation such as in rheumatoid arthritis may cause depletion in vitamin B6. The plasma P5P level in rheumatoid arthritis patients was 24.7 nmol/L compared to 46.2 nmol/L in a control group [102]. On the other hand, despite the dietary intake in some of these patient groups, the deficiency still occurred. Finally, a metabolic abnormality is most likely to occur, either due to decreased formation (inhibition of pyridoxal kinase) or increased degradation

(increased alkaline phosphatase and/or less protein binding). This last mechanism has never been evaluated in this patient population.

Small bowel transplant patients are at the highest risk for developing vitamin B6 deficiency. The hypothesis that this organ transplantation is associated with stronger immune response and so elevated inflammatory markers like alkaline phosphatase and cytokines is expected in small bowel transplant patients. We predicted a higher urinary 4-PA, higher serum alkaline phosphatase, higher cytokine levels and/or lower serum albumin in small bowel transplant patients than in healthy subjects that may contribute to the observed low vitamin B6 levels. This study was designed to quantify the urinary excretion product 4-pyridoxic acid (4-PA) in small bowel transplant patients after transplantation at two time periods. The levels of 4-PA were measured using validated HPLC method. Plasma Alkaline phosphatase and serum albumin were also measured in the central laboratory as part of routine clinical care. Selected plasma cytokines was also measured at different follow up visits as biomarkers of interest.

3.2 CLINICAL STUDY

All plasma and urine samples were obtained from small bowel transplant patients who had participated in a study that was approved by the University of Pittsburgh Institutional Review Board and had provided informed consents for the collected samples to be used in additional investigations.

3.2.1 Clinical Study Design

The primary study was performed in small bowel transplant patients and a healthy control group with no renal or hepatobiliary diseases. *The inclusion criteria included:* age between 18-65, with body weight within 30% of ideal body weight, patients who received small bowel transplantation, and signed the IRB approved informed consent. Transplant candidates were excluded if they had a history of previous organ transplant, a creatinine clearance less than 30 ml/minute, hemoglobin less than 8.5 gm/dL, or were smokers. Any patient on known pyridoxal kinase inhibitor was also excluded. These include: R-roscovitine, 4-deoxypyridoxine, cycloserine, D-penicillamine, dopamine, isoniazid, levodopa, muzolimine, progabide, thiamphenicol and theophylline. All supplemental vitamin B6 intakes were recorded. Transplanted subjects were not studied after transplant if they required supplemental oxygen, were receiving a non-standard immunosuppression protocol or had a clinical or histological evidence of acute or chronic rejection.

Control subjects were age and gender matched healthy subjects. The inclusion criteria were age between 18 and 65 years, weight within 30 percent of ideal body weight, and normal renal and hepatic function. Subjects were excluded if they were smoking, or have a history of intestinal diseases like Crohn's disease.

Study design: Transplant subjects underwent two separate study sessions, one in the early post- transplant period while patients were still in the hospital after surgery and one four to fourteen months after transplantation. Cumulative urine samples (~10 ml) from 0-12 hours were collected after transplantation and the total volume was recorded. Similar collections from a group of healthy volunteers were also performed. The urinary concentrations of 4-PA was

measured using HPLC. The amounts were then calculated given the volume of urine, as (volume x concentration). Alkaline phosphatase and albumin serum concentrations were recorded throughout the study as part of routine clinical care. Blood samples were collected across the study time for selected cytokine analysis

Statistical analysis: Sample size calculation for power of 80% and level of significance of 0.05 was calculated depending on the reported concentrations of P5P in intestinal transplant population before and after transplantation [9]. P5P concentrations were 14.6±13.5 ng/ml and 2.42±0.08 ng/ml, before and after transplantation, respectively. Assuming two independent samples and 1:1 ratio patient to control, the sample size calculated (for power of 80% and level of significance of 0.05) is 22 (11 in each group). The effect size is estimated to be 1.28 and can be utilized to measure the strength of relationship between the treatment and control group. (G Power 3.1.0, 2009)

All data was plotted and tested for normality before further statistical analysis. The 12-hour cumulative amounts of 4-pyridoxic acid were normally distributed over the time interval. The average cumulative total 4-PA excreted in urine over 12 hours was compared between control group and the transplant groups using analysis of variance (ANOVA) with α level of 0.05. Urinary 4-PA was compared between groups with control for different confounding variables like gender and parenteral status (PN). Similar, comparisons using ANOVA were carried out for alkaline phosphatase, albumin and each of the measured cytokines. Subgroup analysis was pursued by dividing transplant group into an early transplant session (<30 days) or Late transplant group (>30 days). The sub-group analysis was basically a t-test for these subgroups and the control keeping the same level of significance as 0.05. In addition, *post-hoc*

Bonferroni correction test was used to compare the two-tailed p-values with the corrected reduced α value between different groups. The new level of significance used is α/n.

3.2.2 Subjects

Transplant recipients were recruited prior to transplant, either in the outpatient transplant clinic or as inpatients during hospital admissions not related to transplantation. Twelve small bowel transplant recipients underwent study session 1 between post-transplant day 10 and 40, six subjects of the session one had complete 12 hour urine collection with volumes recorded. Out of the six transplant subjects who underwent study session 1, two were men and four were women. Seven subjects returned for the second study session and only six had complete 12 hour urine collection (two males and four females). Sixteen healthy age-gender matched control subjects participated in the study, however four of them were excluded from analysis as they were on multivitamin supplement. All transplant recipients were Caucasians. None of the study subjects, controls or transplant, were taking known pyridoxal kinase inhibitors at the time of study. However, some of the transplant subjects were taking some type of supplementation or receiving parenteral nutrition (PN). All transplant patients had tacrolimus as their primary immunosuppressant. Of note, all transplant patients were taking proton pump inhibitor during the study sessions (lansoprazole, pantoprazole or omeprazole were given, typically once per day). All transplant subjects required corticosteroid treatment as methylprednisolone, hydrocortisone or prednisone administered through different routes at some point either during or after transplant. Transplant subjects received nystatin swish and swallow for candida esophagitis prevention, sulfamethoxazole / trimethoprim 400/80 mg tablet by mouth three times a week for

prevention of Pneumocystis carinii (PCP) pneumonia and either oral or intravenous ganciclovir for cytomegalovirus (CMV) prophylaxis.

All transplant patients received other medications, including antihypertensives, antidepressants, and warfarin. Table 17 summarizes the demographics of the participants included in the analysis of this study in both transplant and control groups.

Table 17. Demographics of the participating groups in the clinical study

Number of Participants	Control (n=12) Transplant (n=12, 6 each session)
Age	Control (21-56 Years) Transplant (23-60 Years)
Gender	Control (M=5) Transplant (M=5)
Multivitamin intake	Control (n=0) Transplant (n=3) (PN)
TPN	Control (n=0) Transplant (n=3)
Post Operation Day (POD)	Transplant 10-40 days (n=6) 125-428 days (n=6)

3.2.3 4-Pyridoxic acid measurement in urine

Urinary 4-Pyridoxic acid was measured using the validated HPLC assay developed in our laboratory (previously described) with convenient sensitivity and specificity according to FDA guidelines for validation of bio analytical assays. Urine samples were collected up to 12 hours Calibration curve was constructed using the concentrations (0.0125 – 0.8 µM). The calibration curve was prepared from a pooled urine samples that was treated similarly to the samples from both control subjects and patients.

3.2.4 Results and Discussion

Higher amounts of urinary 4-PA indicated more degradation of P5P and increased excretion as a mechanism of vitamin B6 deficiency. Higher plasma alkaline phosphatase levels observed are expected to be responsible for increased degradation of P5P. Lower albumin levels indicated more of free P5P in plasma that could have facilitated increased degradation as well. Individual levels of selected cytokines during different stages of transplantation highlighted the inflammatory component that was evaluated further.

4-PA cumulative amounts were plotted to see how the values are distributed. Most of the 4-pyridoxic acid urinary excreted amounts were centered around the mean as shown in the following graph. However, the small number of healthy volunteers is a limitation for the test of normality. Normal 4-PA urinary excretion is >1.5 µmol/12hr the distribution is shown in Figure 5

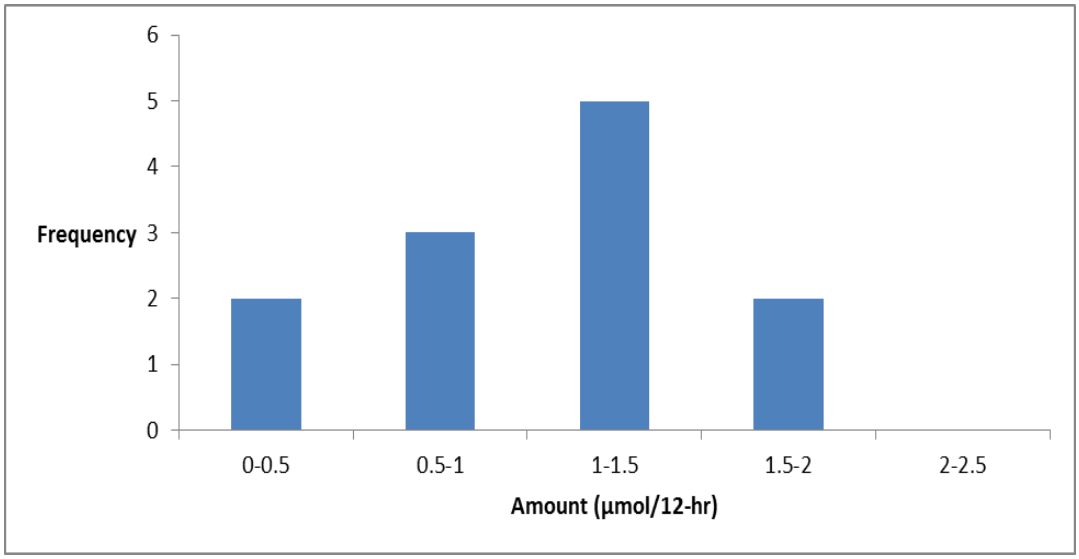

Figure 5. Distribution of urinary 4-pyridoxic acid in healthy volunteers (n=12)

The Cumulative 12-hr Urinary 4-PA in control was (1.11 µmol± 0.13) compared to that in transplant group (16.92 µmol±3.58). Large variation in the transplant group was observed compared to the control group. Single Factor ANOVA is used to compare groups, and the p-value is <0.05 between small bowel transplant group and the control group (Figure 6). This is the first report to our knowledge that documents increased urinary excretion in the small bowel transplant patients.

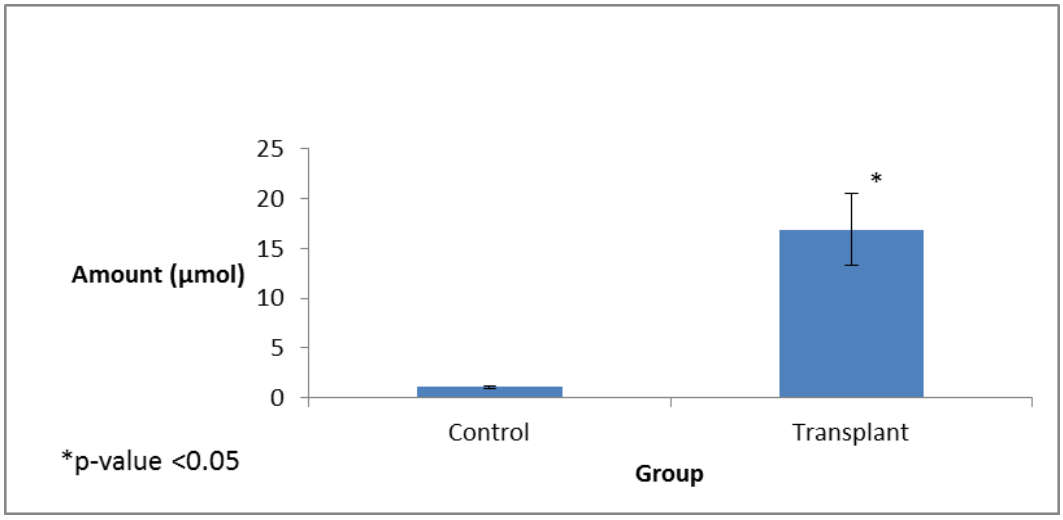

Figure 6. Cumulative 12-hr urinary 4-pyridoxic acid in control (n=12) and transplant (n=12) groups

For further analysis the transplant group was divided into two distinct groups according to their post-operative days (POD). Transplant session 1 was for all patients for whom their POD is less than 30 days and Transplant session 2 was for all patients whom their OD is more than 30 days. The variation in results was the highest during the transplant session 1. Amounts of 4-PA excreted were (1.11 µmol ±0.13), (23.78 µmol ±5.96), and 10.1 µmol ±1.4) in control, group at transplant session 1 and at transplant session 2, respectively. The Statistical analysis using single factor ANOVA between groups showed statistically significances. Then the post-hoc Bonferroni correction test was used to compare the two-tailed p-values with the corrected reduced α value between different groups. The new level of significance is α/n =0.0167. The comparison between control group and either group of transplant sessions showed a significance difference (C vs. TX1 p-value< 0.0167), (C vs. TX2 p-value<0.0167). In addition, there was not a statistically

significant difference between the transplant session 1 and the transplant session 2 (p-value > corrected α= 0.0167) (Figure 7). The analysis suggests that there is significantly higher urinary excretion of 4-pyridoxic acid following transplantation in small bowel transplant patients. This effect was obvious at early session following transplantation; however, the higher urinary excretion of 4-pyridoxic acid was also maintained at the late post-transplant session. This might be explained by continuously increased degradation of P5P following transplantation that may be sustained long term after transplantation.

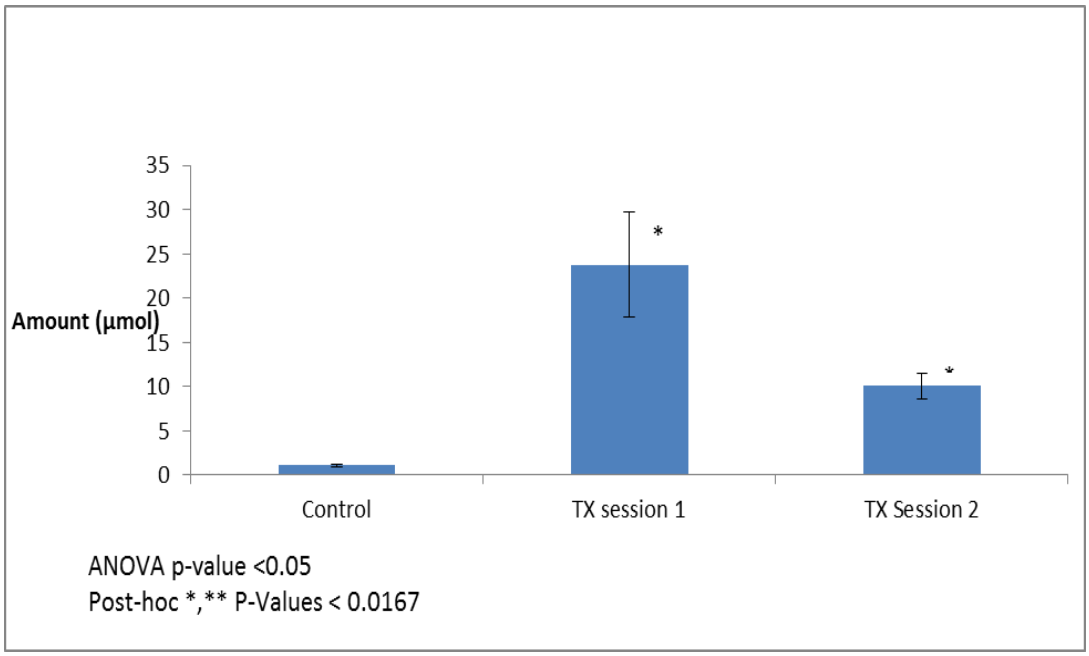

Figure 7. Cumulative 12-hr urinary 4-pyridoxic acid in control (n=12) and transplant groups (n=6)

The transplant group and the control group consisted of 7 females and 5 males. As expected there was no statistical difference between males and females in the control group. In

control groups, the mean value in females was (1.11 µmol±0.21) vs. (1.1 µmol±0.0.11) in males. ANOVA single factor found a significant difference between all groups control males, control females, transplant females, and transplant males (p-value <0.05). The post-hoc analysis using Bonferroni correction revealed no statistically significant difference between gender subgroups. The results in transplant group also was not statistically significant, however there were overall higher levels of 4-PA in females as well as higher variation (F 20.33 µmol±5.93, M 12.15 µmol±0.9). The two-tail p-value was > 0.0167. The National Health and Nutrition Examination Survey 2003-2004 (NHANES) (n=9000) indicated that plasma P5P concentrations of women of childbearing age (n=2048) were significantly lower than those of comparably aged men (n=2351), especially in women using oral contraceptives. These were more prevalent in subgroups of smokers, elderly, non-hispanic blacks, and current and former oral contraceptive users. The gender differences in vitamin B6 status were attributed to estrogen intake that might interfere with P5P dependent tryptophan metabolism [8].

Parenteral Nutrition (PN) status is not uncommon among the small bowel transplant patients and can be a confounding factor in interpretation of this result. A comparison among the transplant subjects controlling for their PN status confirmed that higher 4-PA urinary amounts were observed among subjects receiving PN. The 4-PA levels were statistically significant higher in patients receiving PN accompanied by a higher variation (PN 36.9 µmol± 2.4, No-PN 10.7 µmol±0.93), p-value <0.05). However, the number of patients who were on PN after transplantation was low (n=3) in comparison to patients who were not on PN. The confounding effect of PN can be tested with larger sample size analysis on both groups.

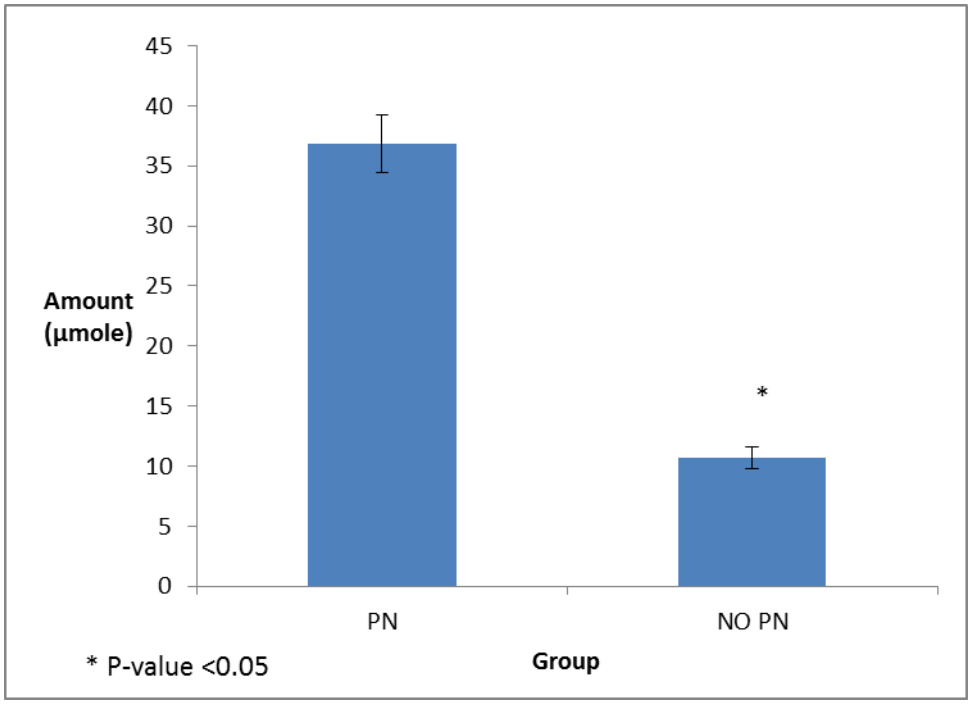

Figure 8. Cumulative urinary 4-PA amount in transplant groups according to PN status

The control group (1.11 µmol± 0.13) and the transplant group not receiving PN (10.28 µmol± 0.93) were compared by Single factor ANOVA and the comparison showed a statistical difference regardless of PN status. The conclusion is that the 4-pyridoxic acid urinary levels are not as a result of supplementation with PN; rather it is an ongoing process that promotes degradation independent of intake.

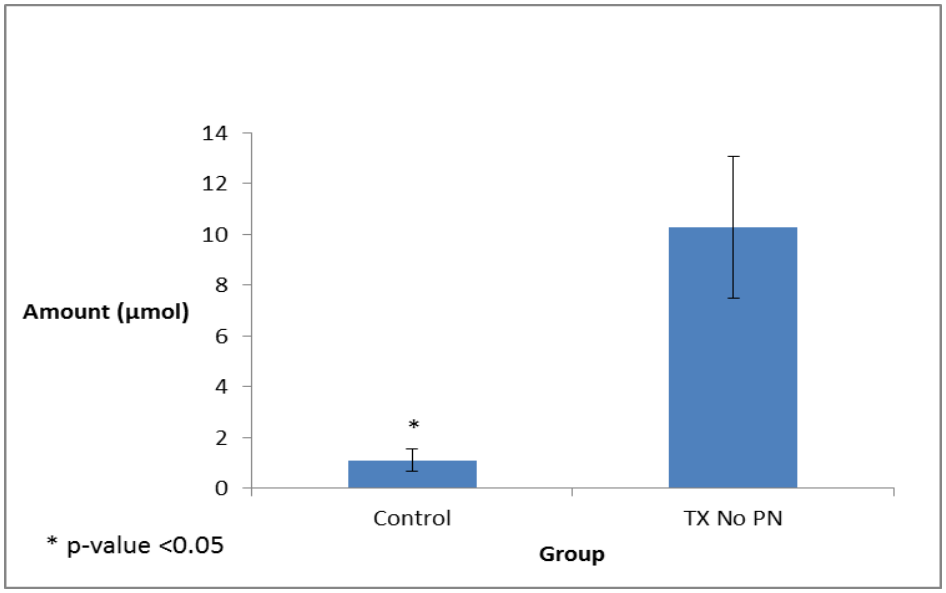

Figure 9. Cumulative urinary 4-PA amount in control and transplant groups controlling for PN status

This graph suggested that increased 4-pyridoxic acid excretion is predominant following small bowel transplantation regardless of intake. Many patients of small bowel transplant patients will be given TPN for variable time periods in order to regain the functional status of the transplanted organ.

Limitations: The study had limited number of subjects who participated. The urine collection for extended period of time with the volume recording was a challenge to get a complete set of urine samples for 12-24 hours. The variability in patient clinical condition, medication regimen as well as individual variation is also another confounder factor. All analysis was carried out by assuming the normal distribution, which is hard to document, given that the sample size is relatively small. However, the developed validated HPLC assay served as a powerful analytical tool to track the metabolic product of vitamin B6.

3.2.5 Alkaline Phosphatase

The normal levels of alkaline phosphatase in normal healthy adults range between 30 – 140 IU/L. This inflammatory marker is measured regularly to monitor for signs of acute rejection and is of special interest in our case as it accelerates the degradation of Pyridoxal 5 Phosphate (P5P) into its ultimate urinary metabolite 4-PA. Average alkaline phosphatase (ALKP) was (56.8 IU/L ±3.7) for control group and (112.5 IU/L ±18.4) for transplant group. Even though average levels in both groups are within normal range, they were statistically significantly different (p-value < 0.05) (Figure 10).

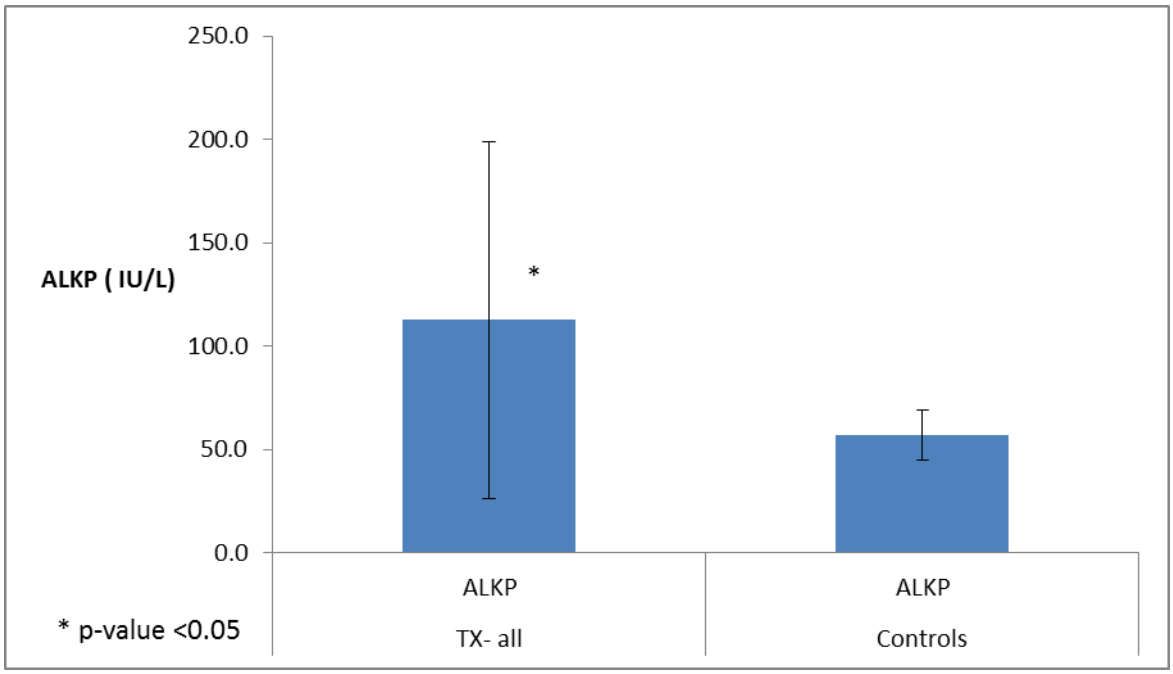

Figure 10. Plasma alkaline phosphatase levels in control (n=12) and transplant group (n=12)

Group comparison between different transplant groups as per session and control group using single factor ANOVA showed no statistically significant difference (p-value=0.052). Also, the comparison between transplant sessions and control group showed a statistical significance between transplant session 2 and control group (p-value< 0.05). The session 1 transplant group patients alkaline phosphatase is statistically significant from control at α=0.05 (p-value < 0.05). However, with post hoc analysis with Benferroni correction there is not a significant difference between both groups. Finally, there is no statistically significant difference between transplant sessions in terms of alkaline phosphatase levels (Figure 11).

Control vs Transplant	NS
Control vs Transplant session 1	P<0.05
Control vs Transplant session 2	P<0.05
Transplant session 1 vs Transplant session 2	NS

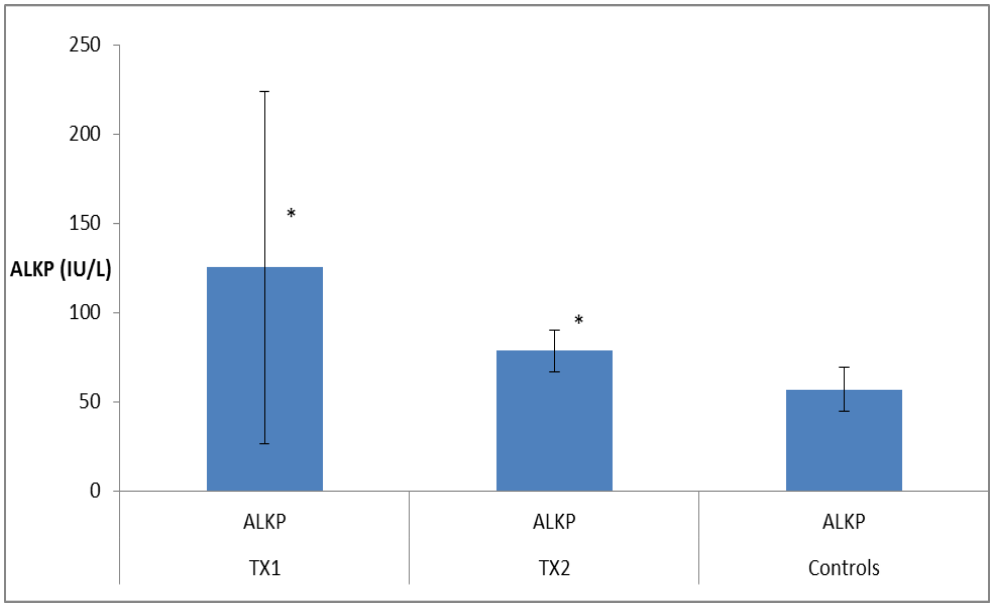

Figure 11. Alkaline phosphatase levels in control (n=12) and different transplant groups (n=6 each)

Abnormal vitamin B6 metabolism has been reported in alkaline phosphatase knock-out mice. These animals eliminated the active coenzyme P5P into urine which strengthen the hypothesis that alkaline phosphatase plays an important role in the metabolism of P5P [103]. The increased levels of alkaline phosphatase seem to shift the metabolism towards more degradation of P5P into the inactive metabolite (4-pyridoxic acid). These finding of elevated alkaline phosphatase after transplantation that is sustained until late session might explain the increased degradation of P5P into 4-pyridoxic acid. Hydrolysis of pyridoxal 5` phosphate (P5P) in plasma was demonstrated in patients with conditions of elevated alkaline phosphatase and some hepatic abnormalities. This study inferred an inverse relationship between the plasma levels of Pyridoxal

5` phosphate and the alkaline phosphatase levels (r=0.893, p-value <0.001). This study provides evidence that vitamin B6 deficiency might be explained by increased degradation of pyridoxal 5` phosphate by elevated levels of alkaline phosphatase [104].

Limitations: As alkaline phosphatase is an inflammatory marker that can be elevated in many conditions and can be variable in activity depending on the source. In our study, we measured plasma alkaline phosphatase regardless of its source. The relationship between elevated alkaline phosphatase and increased degradation of pyridoxal 5' phosphate has been suggested, however the study design being observational in nature cannot conclude the causality. An alkaline phosphatase knock out animal can serve as a model to evaluate the exact role of alkaline phosphatase in the increasing degradation of P5P.

3.2.6 Serum Albumin

The normal range of albumin in healthy adults is 3.4 – 5.4 g/dl. Pyridoxal 5'Phosphate (P5P) is predominantly bound to albumin in plasma and only the unbound P5P will be subject to alkaline phosphatase mediated further degradation into ultimate 4-PA urinary metabolite. Average albumin level was compared between control group and transplant group. Albumin levels were measured frequently following transplantation and serum samples were collected for albumin measurement. The laboratory results for these transplant subjects were extracted from the patient chart. The control subject serum albumin were ordered and recorded as the comparison group. Average serum Albumin was (4.0 g/dl ±0.14) in the control group and (3.4 g/dl ±0.08) in the transplant group. Though levels in both groups were within normal range, they were statistically significantly different from one another (p-value < 0.05) (Figure 12).

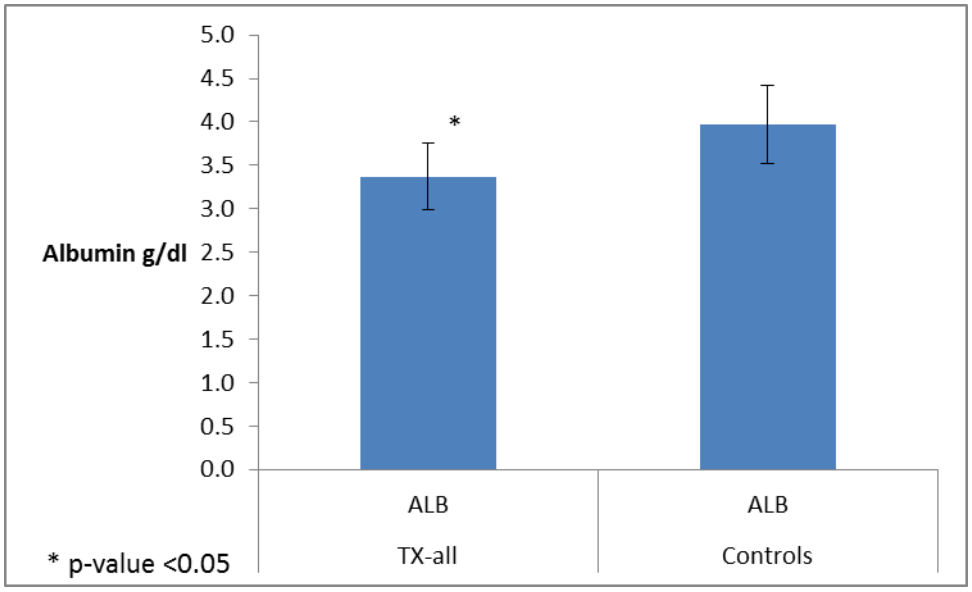

Figure 12. Serum albumin levels in control (n=12) and transplant group (n=12)

Single factor ANOVA showed a statistically significant difference between groups (control subjects, transplant session 1 and transplant session 2), p-value <0.05. Furthermore, subgroup comparison using the post-hoc Bonferroni correction between different transplant groups and control group revealed a statistically significant difference between transplant session 1 and transplant session 2 and the control group with p-values <0.0167 (Figure 13). Albumin status is very important for the distribution of the active vitamin B6 form (pyridoxal 5` phosphate). Low levels of albumin may increase the free fraction of pyridoxal 5` phosphate which makes it more prone to degradation by alkaline phosphatase and aldehyde oxidase.

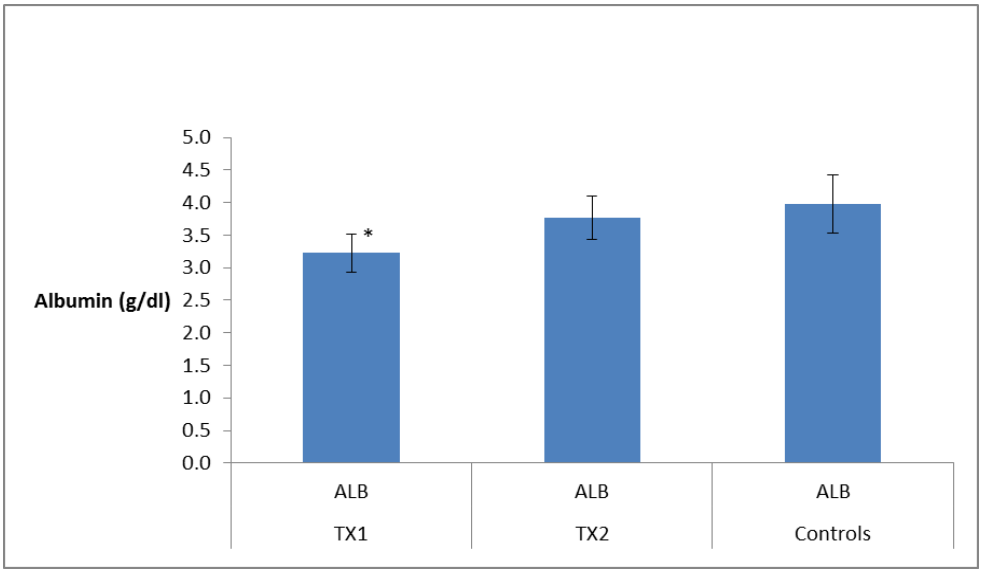

Figure 13. Serum albumin levels in control (n=12) and different transplant groups (n=6)

Lower serum albumin levels, the main binding protein for the active P5P in plasma, may result in the active form being more prone to degradation by alkaline phosphatase, which is also elevated. This can account for the increased degradation of P5P into 4-pyridoxic acid. The levels of albumin in small bowel transplant were lower than in that of control subjects despite the fact that they are all within normal range. The extent of this possible effect is difficult to quantify but it could be another contributing factor to the observation of increased degradation product in the urine. A study that investigated the increased hydrolysis of pyridoxal 5` phosphate due to increased alkaline phosphatase in different non-transplant hepatobiliary disorders measured serum albumin as the main binding protein to P5P. Interestingly the hydrolysis rate in one hour did not bear any relationship to the albumin levels, which ranged from 26g/L to 47g/L [104].

Limitations: albumin is an endogenous compound that is affected by many variables including liver function, trauma, and medications. The levels of albumin can vary extensively

throughout the course of study in these patients due to their clinical status as well as their dietary intake. However, the overall effect was expected and that suggested a probable role for low albumin levels in increasing the degradation of P5P.

3.2.7 Cytokine Analysis

Cytokines are functional proteins that are mainly produced by immune cells like macrophage and lymphocytes as well as other cells like epithelial cells and smooth muscle cells. In organ transplantation, cytokines are indicators for acute phase response and organ rejection. Many cytokines are known to have an effect on activity and expression of some metabolizing enzymes and/or transporters. For example: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL12, TNF-α, IFN-γ [105, 106, and 107]. Table 18 summarizes the effect of different cytokines on metabolic enzymes and or transporters.

Table 18. Cytokines effect on metabolism and normal ranges

Cytokine	Effect on Metabolism/ Transporter	Normal range
IL-1β	CYP3A4,MRP(2,3,4),BCRP, OATP1, MDR1	0.96 ± 0.12 pg/ml
IL-2	MDR1	0.3 ± 1.4 pg/ml
IL-4	NA	1.4 ± 5.4 pg/mL (range 0-27)
IL-6	CYP3A4, CYP1A2, CYP2C19	1.2 ± 2.4 pg/mL
IL-8	NA	9.76 ± 1.31 pg/mL
IL-10	NA	6.4 ± 15.0 pg/mL
IL-12	NA	of less than 10 pg/mL
TNF-α	CYP3A4,MDR1, CYP2C19	1.3 ± 2.6 pg/mL
IFN-γ	CYP3A	of less than 5 pg/mL

Blood was withdrawn from control subjects once and at several time points post small bowel transplantation and aliquots were assayed for selected human cytokines: interleukin-1β (IL-1 β), interleukin-2 (IL-2), interleukin-4 (IL4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ).

All assays were carried out at Luminex core facility. These cytokines were measured in plasma using a MILLIPLEX MAP High sensitivity Human Cytokines Multiplex Panel kit (# HSCTTO-60SK, Millipore, Billerica, MA). Reagents included in the kit were a high sensitivity human cytokine standard, high sensitivity human cytokines quality controls 1 and 2, serum matrix containing 0.08% sodium azide, 96-well filter plate with sealers, 10X wash buffer containing 0.05% Proclin, high sensitivity human cytokine detection antibodies, and streptavidin-phycoerythrin solution. The bead based multiplexing assay is simply where the beads have fluorescent dyes that will produce a specific spectral address upon conjugation with the analyte of interest. This technology, although very expensive, allowed multiple detections of

several cytokines with a very small sample volume. Each individual cytokine average was compared between transplant and control group. There were a total of 80 blood samples for analysis including control samples and the rest were a mix of session 1 and session 2 transplant subjects. Two plates were run to accommodate all samples.

Frozen plasma samples were thawed, mixed, and centrifuged to remove particulates. Following the manufacturer directions the standard curves were constructed using quality controls and serum matrix to different concentrations (0.13, 0.64, 3.2, 16, 80, 400, and 2,000 pg/ml). Twenty five μL of premixed antibody beads were then added to each well then an additional 50 μL of each of the standards and the quality controls were added to the designated wells along with 50 μL of serum matrix. To this whole mix a 50 μL of buffer and the sample are subsequently added. The filled plate is then sealed and incubated overnight on a shaker at 4°C. The different standard curve range for different cytokine is shown in Table 19.

Table 19. Standard curve range for different cytokines

Cytokine	Standard Curve range (pg/ml)	
	Plate 1	Plate 2
IL-1β	0.13 -359.24	0.79-334.65
IL-2	0.13 -2122.24	0.77 -2395.94
IL-4	3.72 -2002.91	3.02 – 2000.03
IL-6	0.14 -2156.73	0.79 – 343.75
IL-8	0.13 -1721.55	0.13 – 2308.77
IL-10	3.25 -2019.26	3.08 -2018.59
IL-12	0.72 -2034.93	0.83 -2050.18
TNF-α	0.13 -2286.95	0.14-366.14
IFN-γ	0.16 – 2010.6	4.15 – 2027.17

The next day, the mixture was removed and the plates were washed with buffer solution. Fifty μL of detection antibodies were added to each well and the plate resealed and incubated at room temperature for an hour on a plate shaker. An additional 50 μL of streptavidin-phycoerythrin was added to each well and similarly incubated for another 30 minutes. Finally, the wells were washed out and all fluids were removed. The beads in the wells were re-suspended by 100μL of sheath fluid.

Finally, the plates were read on a Luminex 100 analyzer (Luminex Corporation, Austin, TX). The Fluorescent intensity (FI) output was analyzed using Bio-Plex Manager software (Bio-

Rad Laboratories, Hercules, and CA). A minimum of five points were used to generate each standard curve. Points were excluded from the curve if the (observed concentration/expected concentration)*100 was less than 70 or greater than 130.

Statistical significant difference between either transplant session and control group are summarized in Table 20. The results were expressed as average observed values ± standard error as the number of samples that was reported in each session for different cytokines were variable. IL-6, IL-8, IL-10, TNF- α were significantly higher during the early transplant session when compared to controls. Using ANOVA analysis, both IL-6, and TNF-α were significantly higher in transplant patients in both session 1 and 2 than in control group. Both IL-8 and IL-10 were significantly higher than in control during transplant session 1. The rest of the cytokines were not significantly different from control group.

Table 20. Plasma cytokine analysis shown as average ± standard error in transplant different sessions and control subjects

Cytokine	Transplant Session 1 Obs. Conc. ±SE (pg/ml)	Transplant Session 2 Obs. Conc. ±SE (pg/ml)	Control Obs. Conc. ±SE (pg/ml)	P-value Single Factor ANOVA
IL-1β	0.90 ±0.17	0.78 ± 0.13	1.18 ± 0.26	0.43
IL-2	0.74 ±0.11	0.81 ± 0.11	0.70 ±0.19	0.17
IL-4	7.2±1.40	8.24±0.83	6.18±1.79	0.63
IL-6	14.0±2.32	4.93 ±1.52	1.27±0.17	**0.003**
IL-8	20.6±2.4	4.81±1.39	1.68±0.13	**<0.0001**
IL-10	57.9 ±5.3	10.74±2.40	5.84±0.76	**<0.0001**
IL-12P70	2.3 ±0.60	3.32 ±1.52	1.46±0.36	0.29
INF-γ	7.6 ± 2.8	5.85±2.67	3.54 ± 0.65	0.54
TNF-α	7.2±0.83	3.11±0.30	1.43±0.15	**<0.0001**

The following series of graphs is a representation of different cytokines in both transplant sessions and control group. IL-6 and TNF-α were significantly higher than control groups in small bowel transplant patients in both transplant sessions. IL-6 average plasma concentration is significantly higher than that in control subjects. Single factor ANOVA analysis was significant in between groups analysis ($p<0.05$). The post-hoc Bonferroni correction confirmed the statistically significance difference between session 1 and the control group ($p<0.0167$). The

comparison between session 2 and the control group is only statistically significantly different at p-value <0.05**.** The comparison is shown in Figure 14. And, the "*" denotes the statistically significant difference.

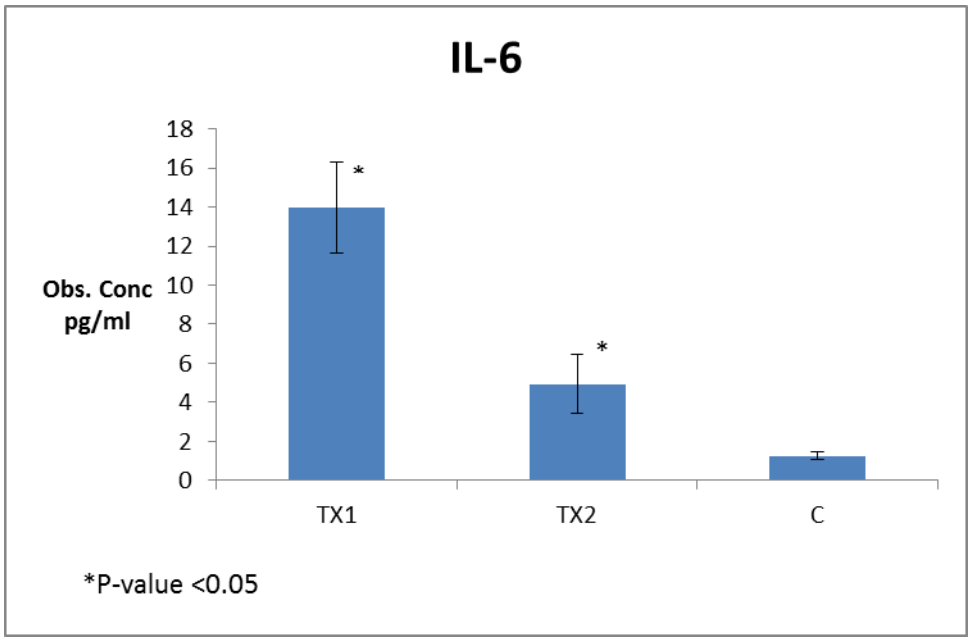

Figure 14. IL-6 mean plasma concentration in control (n=12) and different transplant groups (n=6 each)

TNF-α average plasma concentration is significantly higher than that in control subjects. Single factor ANOVA analysis was significant in between groups analysis ($p<0.05$). The post-hoc Bonferroni correction confirmed the statistically significance difference between both session 1 and session 2 when compared to control subjects (p-value <0.0167). The comparison is shown in Figure 15 and, the "*" denotes the statistically significant difference.

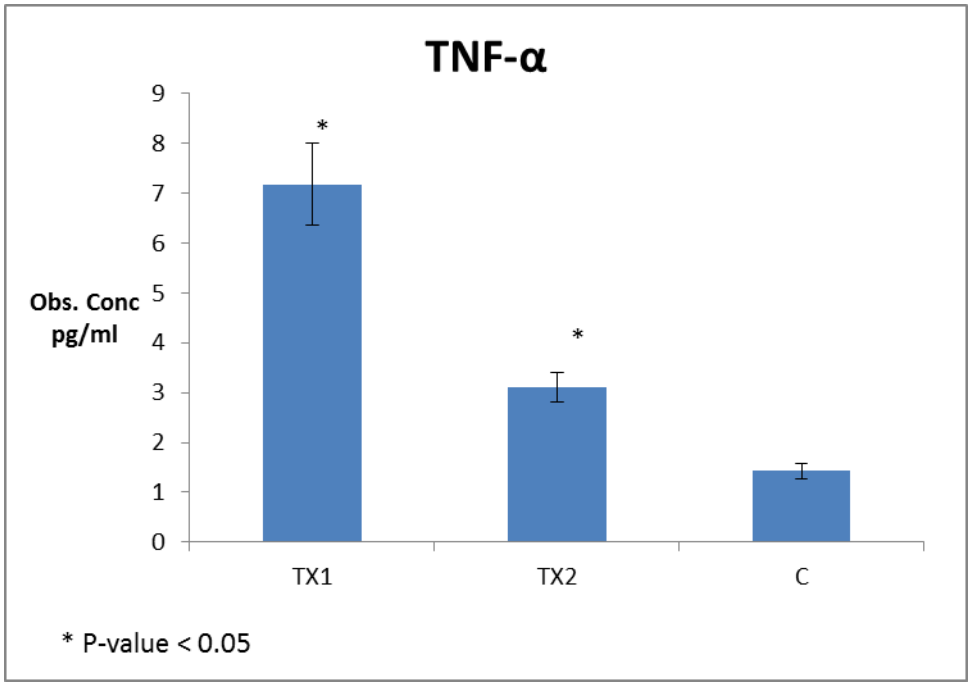

Figure 15. TNF-α mean plasma concentration in control (n=12) and different transplant groups (n=6 each)

The following cytokines showed a statistical significance difference between transplant session 1 group and control group, these are IL-8 and IL-10. IL-8 average plasma concentration is significantly higher than that in control subjects. Single factor ANOVA analysis was significant in between groups analysis ($p<0.05$). The post-hoc Bonferroni correction confirmed the statistically significance difference between only session 1 and the control subjects (p-value <0.0176). The comparison is shown in Figure 16 and, the "*" denotes the statistically significant difference.

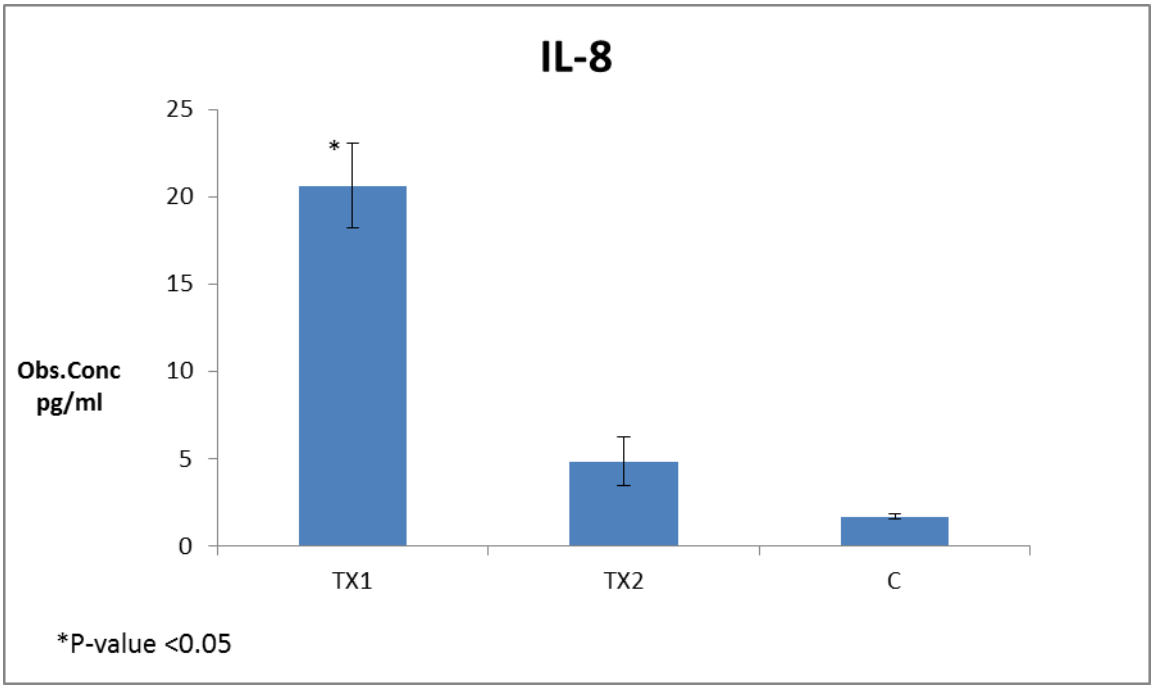

Figure 16. IL-8 mean plasma concentration in control (n=12) and different transplant groups (n=6 each)

IL-10 average plasma concentration is significantly higher than that in control subjects. Single factor ANOVA analysis was significant in between groups analysis ($p<0.05$). The post-hoc Bonferroni correction confirmed the statistically significance difference between session 1 and control subjects (p-value <0.0167). The comparison is shown in Figure 17 and, the "*" denotes the statistically significant difference.

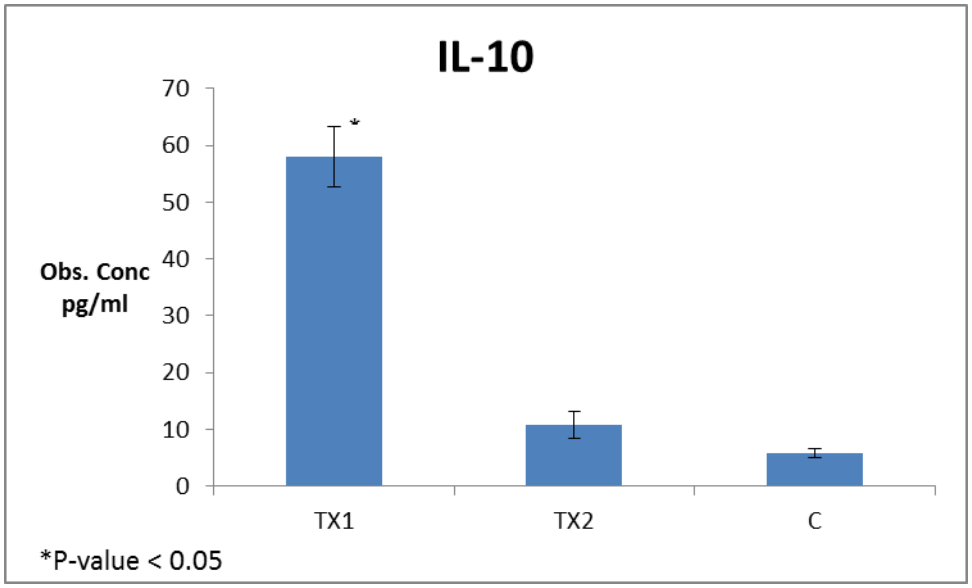

Figure 17. IL-10 mean plasma concentration in control (n=12) and different transplant groups (n=6 each)

The rest of cytokines showed no statistical difference between either transplant groups or the control group, and this group includes: (IL-1β, IL-2, IL-4, IL-12p70, and INF-γ). Single factor ANOVA analysis was not significant in between groups analysis ($p>0.05$). The post-hoc Bonferroni correction confirmed there is no statistically significance difference between both session 1 and session 2 when compared to control subjects. NS in the graphs stands for not significant difference.

Limitations: the assay for cytokine analysis is labor intensive and expensive that limited the types of cytokines measured. However, the nine cytokine panel measurement was convenient and feasible as it includes almost all commonly reported cytokines in different transplant patients. The technique used based on Multiplexing that allows the measurement of all cytokines

in one sample at the same time. Further studies might focus on the notably elevated cytokines which might be better option to utilize resources.

3.3 CONCLUSIONS

In recent study by Matarese *et al.*, the P5P deficiency occurred in 96 % of small bowel recipients within a median of onset of 30 days (range 4-118 d) after transplantation. About 41% of these patients received parenteral nutrition. The main finding was undetectable P5P concentration. This finding explained the clinical observations of unexplained neuropathy or myopathy in a few of long-term survivors. However, underlying cause for such a deficiency was not clearly identified. The authors suggested an increased metabolic demand along with drugs used for immunosuppression as a cause of the observations [9].

This clinical study reveals a possible mechanism for the biochemical deficiency of vitamin B6 post transplantation in small bowel transplant population. The increased urinary metabolite 4-pyridoxic acid suggested strongly that more degradation of the active form pyridoxal 5' phosphate is occurring. This was supported by the elevated levels of non-specific alkaline phosphatase throughout the study period. As the active form need to lose its phosphate group in order to get converted to its ultimate urinary 4-pyridoxic acid by aldehyde oxidase. The elevated urinary 4-pyridoxic acid in transplant group in early session and even few months after transplantation was the main finding in this clinical study. Merrill *et al*. suggested that vitamin B6 deficiency in patients with cirrhosis was attributed to increased dephosphorylation e.i degradation rather than altered formation of pyridoxal 5` phosphate. In addition, the serum

albumin levels in patients with cirrhosis were also lower than the control subjects, which is also similar to our finding regarding lower serum albumin in the small bowel transplant patients [108].

The higher levels of 4-pyridoxic acid in transplant groups is supported by the finding of increased alkaline phosphatase levels in the transplant groups in addition to lower levels in serum albumin. This last factor might lead to increasing degradation level of pyridoxal after getting dephosphorylated by alkaline phosphatase. As less albumin may lead to more of free unbound pyridoxal 5` phosphate that is consequently more available to get dephosphorylated and ultimately degraded by aldehyde oxidase into urinary 4-pyridoxic acid.

In addition, P5P is predominantly bound to albumin in circulation, which gives another layer of protection against degradation. The significantly less than control average albumin among transplant patient – although within normal range- also suggested relatively more free P5P will be subjected to alkaline phosphatase and further degradation into 4-Pyridoxic acid.

The cytokine analysis showed the majority of cytokines are not different from the control healthy groups. The few cytokines that showed elevation following transplantation had no reported influence on pyridoxal kinase activity. However, IL-6 and TNF-α were significantly higher in transplant groups than in controls and only IL-8 and IL-10 were significantly higher than control in the early session follow-up. The role of cytokines on vitamin B6 status is not clear and will be evaluated. There is a body of evidence that suggested a cytokine effect on different metabolic enzymes like CYP enzymes and some transporters; however, there is no supporting evidence in such an effect on pyridoxal kinase enzyme activity or expression. However, a variety of inflammatory disease conditions have been found to be associated with low levels of plasma pyridoxal 5` phosphate without any indication of a lower dietary intake of

vitamin B6. The Suggested explanation is the mobilization of this co-enzyme (P5P) to sites of inflammation for use in P5P dependent enzymes [109]. Also, vitamin B6 status, based on pyridoxal 5` phosphate levels in plasma was identified in different inflammatory diseases such as cardiovascular diseases, rheumatoid arthritis, inflammatory bowel disease, and diabetes. A study in a population of U.S. adults (n=2229) suggested a strong inverse relationship between P5P levels and some inflammatory markers including cytokines such as IL-6 and TNF-α [110].

In summary, our prediction of increased urinary excretion of 4-PA was confirmed. The proposed mechanism was attributed to increased alkaline phosphatase in plasma following small bowel transplantation. As the degradation of P5P was likely promoted by increased alkaline phosphatase levels, this reflected by higher urinary 4-PA excretion in small bowel transplant subjects. In addition, the lower albumin levels in small bowel transplant subjects may lead to increased free fraction of P5P, that likely lead to more degradation of P5P free fraction by alkaline phosphatase. Finally, the blood analysis revealed elevation of some pro-inflammatory cytokines that might have a role on P5P metabolism.

4.0 COMPUTATIONAL APPROACH TO PREDICT DRUG INTERACTIONS WITH PYRIDOXAL KINASE

Abstract:

Small bowel transplant patients on an average receive 12 different medications. There is always a high probability for drug-drug interactions or drug-enzyme interactions. A computational approach to test the effect of complex regimen of medications on the activity of an enzyme involved in vitamin B6 metabolism was designed and carried out. The crystal structure of pyridoxal kinase enzyme was imported into the computer and the spatial binding sites were identified. All different drugs were also saved into a format compatible (SDF) for the operating system. The docking score was the primary outcome of this fitting exercise where the higher the docking score is the more likely the drug will spatially fit and the interaction is more likely to occur. For the validation purposes, known inhibitors were also tested under the same programming conditions. The scores were recorded and the probable high score compounds were further tested *in vitro* to confirm the results. Pantoprazole, a proton pump inhibitor that was used almost in all small bowel transplant patients, was among the list of drugs with high docking score (7.17) in addition only one immunosuppressive drug (mycophenolic acid) also showed a relatively high docking score (5.2). These two drugs were selected to be further tested in *in vitro* experiments that will be described in the later chapters.

4.1 INTRODUCTION

Small bowel transplant patients usually receive a large number of medications for the rest of their lives. With this complex regimen, medications may play a role in the vitamin status given that there is a group of medication known for their inhibitory effect on pyridoxal kinase, the enzyme responsible for conversion of vitamin B6 into pyridoxal 5' phosphate (P5P), the active form of vitamin B6. These known inhibitors are not typically used in this patient population and include the following: R-roscovitine, 4-deoxypyridoxine, cycloserine, D-penicillamine, dopamine, isoniazid, levodopa, muzolimine, progabide, thiamphenicol and theophylline. On the other hand, the small bowel transplant patients may take 10-15 different medications that have never been tested for their inhibitory effect on pyridoxal kinase. These medications include immunosuppressant, antibiotics, antivirals, antifungals, steroids and others. The median number of daily oral medications was 7 (4 – 11) in study session 1 and the median was 14 (10 -22) in study session 2. These medications were described in Table. 21. We utilized a computational approach to predict potential impact of drugs used in transplant patients described in chapter 3 on the activity of certain enzymes involved in the pathway of vitamin B6 metabolism.

Table 21. Medication used by transplant subjects during study sessions

Medication	**Medication**	**Medication**
Amlodipine	Diphenoxylate / atropine	Cholecalciferol
Metoprolol	Ganciclovir	Calcium citrate + Vitamin. D
Warfarin	Acyclovir	Folic acid
Sertraline	Valganciclovir	Ferrous gluconate
Trazodone	Clonazepam	Zinc sulfate
Mirtazapine	Zolpidem	Riboflavin
Bupropion	Sodium bicarbonate	Methylphenidate
Hydromorphone	Magnesium gluconate	Levitracetam
Loperamide	Pyridoxine	Pravastatin

"Human Pyridoxal Kinase is a homodimer related by a non-crystallographic twofold axis in an asymmetric unit. Each pyridoxal kinase monomer contains nine α-helices (named α1-9), and 12 β-strands (named β1-10 and β-5a, β5b). Human pyridoxal kinase is an elongated dimer with the approximate dimensions of 85 Å x 60 Å x 40 Å. The dimer interface is formed between α1, α9, β1, and β3 from each monomer with a non-crystallographic twofold symmetry through hydrogen bonding, salt bridges, and hydrophobic interactions. The buried area is approximately about 13% of the total surface area in one monomer. The dimer interface has hydrophobic residues Ile-15, Ile-35, met-74, and Met-287 that facilitate a strong hydrophobic interaction

between monomers of pyridoxal kinase. The binding site is a cavity with negative charge located along one edge of the central β-sheet that attracts substrates with positive charge, such as the pyridine ring of vitamin B6 and the adenine ring of ATP. The ATP-binding site is located in a shallow groove formed by the hydrophobic side chains of surrounding residues. On the opposite direction of ATP binding site the pyridoxal-binding site is located." [111].

4.2 METHODS

An innovative computational approach to predict drug interactions with pyridoxal kinase was utilized .The crystal structure of Pyridoxal kinase were simulated into the computer and all medications that would be used by typical small bowel transplant patient were docked and the score were calculated. All known inhibitors (virtually) were used as control for this computational exercise. SYBYL® from Tripos was utilized for receptor ligand modeling. Pyridoxal is the typical substrate for this enzyme that has a pyridoxal binding pocket and an ATP binding pocket where phosphorylation takes place.

4.3 RESULTS

Pyridoxal kinase crystal structure was identified through a known chemical structures library and the PDB ID was 1 RFU where the red color represents α-helices and the blue color

represents the β-strands. Figure 18 is a computer generated snap shot of our enzyme and Figure 19 is another representation that showed the ATP-binding pocket.

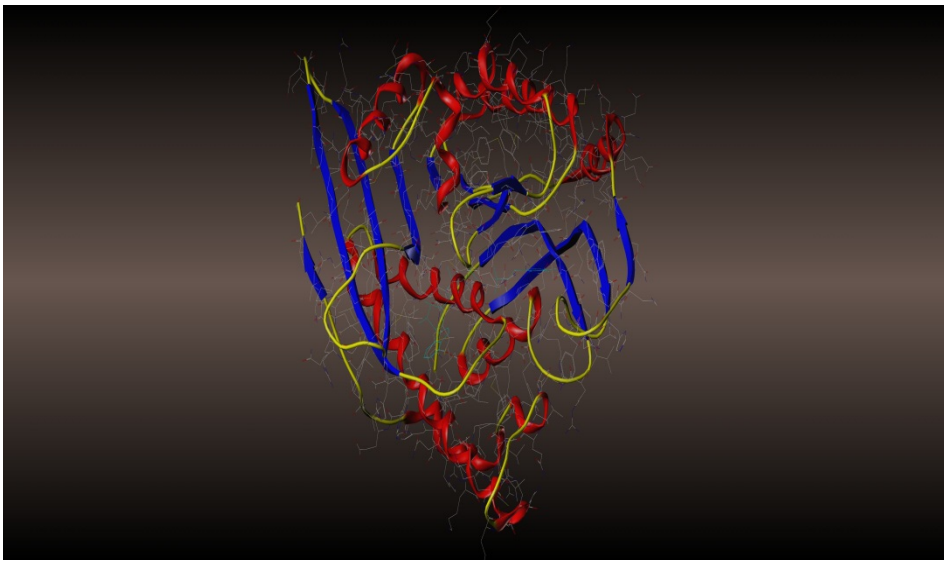

Figure 18. Pyridoxal kinase crystal structure, PDB ID: 1RFU

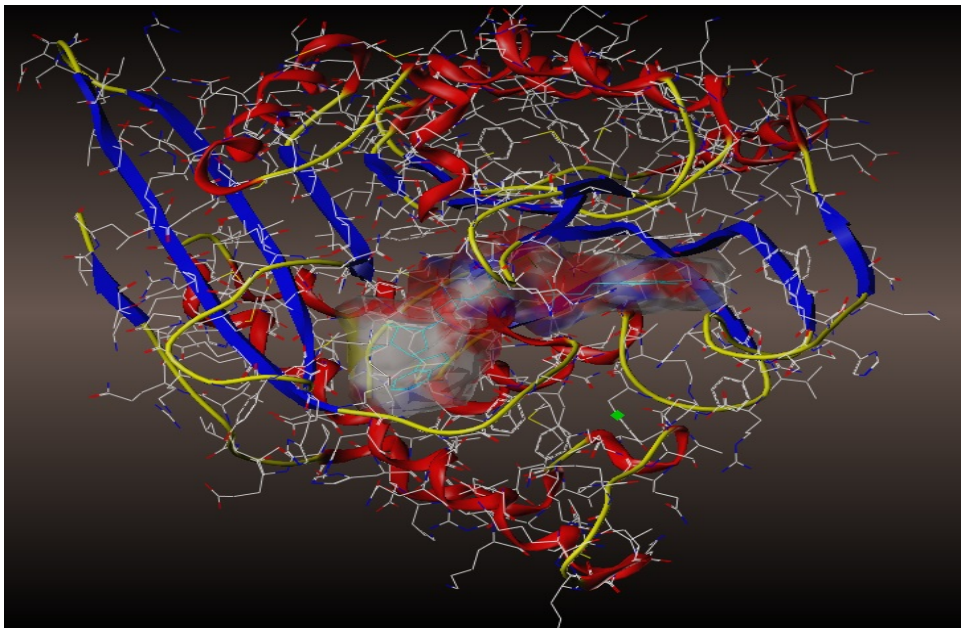

Figure 19. Pyridoxal kinase with ATP-binding pocket

The Known inhibitors were used to validate our spatial configuration and return relatively high docking scores that are shown in Table 22. The lead substrate pyridoxal was used as the reference value (docking score= 3.01) and is shown in Figure 20.

Table 22. Docking scores of known pyridoxal kinase inhibitors

Inhibitor	**Docking Score**
Deoxypyridoxine	**6.41**
Levodopa	5.31
Roscovitine	4.55
Dopamine	4.36
Thiamphenicol	4.16
Cycloserine	4.12
Theophylline	4.05
Muzolimine	4.02
Isoniazid	3.95
Progabide	3.24

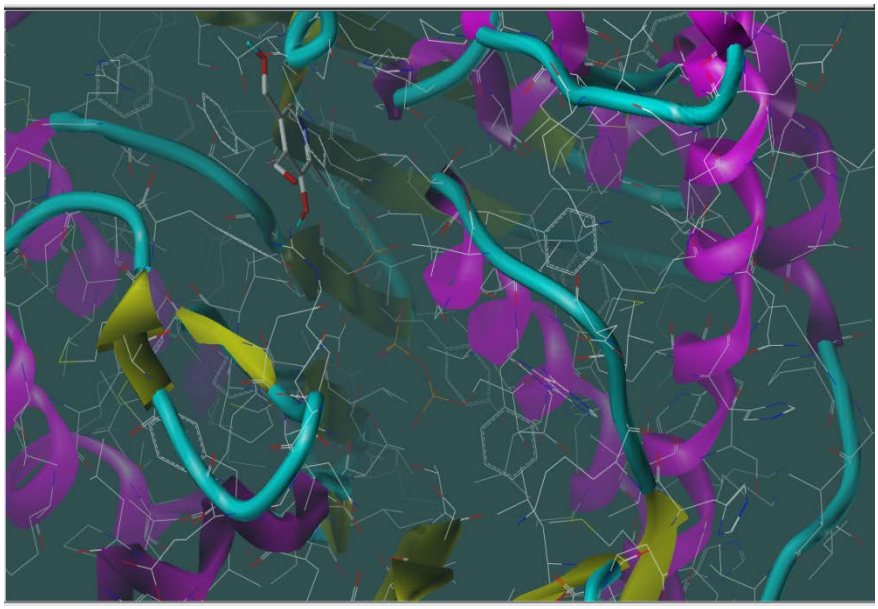

Figure 20. Pyridoxal (Lead substrate), Docking score=3.01

All medication used or may be used throughout the course of their post transplantation were docked and the docking scores were calculated. Compounds like tacrolimus were not likely to score high because of the huge size but other compounds were a finding that granted us a

justification for further testing. The chemical structures of all these compounds were gathered and saved as SDF files that were compatible with the software. The docking scores for all these tested medications are shown on Table 23. Notably, pantoprazole scored the highest (7.17) (Figure 21). Interestingly, the only immunosuppressive drug mycophenolic acid score was 5.2 and all the other immunosuppressive drugs (tacrolimus, sirolimus, and cyclosporine) scored very low (Figure 22). The results with these large molecules were anticipated.

Table 23. Docking score of different drugs used in transplant patients at pyridoxal kinase as target enzyme

Drug	**Docking Score**	**Drug**	**Docking Score**
Pantoprazole	7.17	Voriconazole	3.58
Trimethoprim	6.54	Fluconazole	3.09
Acyclovir	5.48	**Pyridoxal**	3.01
Valganciclovir	5.44	Prednisone	2.65
Mycophenolic acid	5.2	Methyl prednisone	2.38
Ganciclovir	4.91	**Tacrolimus**	-15.52
Ranitidine	4.87	Nystatin	-81.92
MMF	4.79	Sirolimus	-133.32
Sulfamethoxazole	4.33	Cyclosporine	-389.80

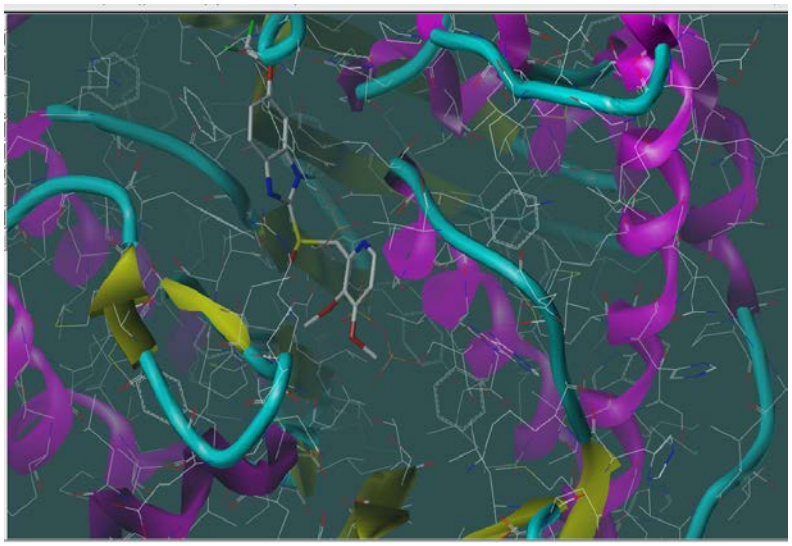

Figure 21. Pantoprazole, docking score=7.17

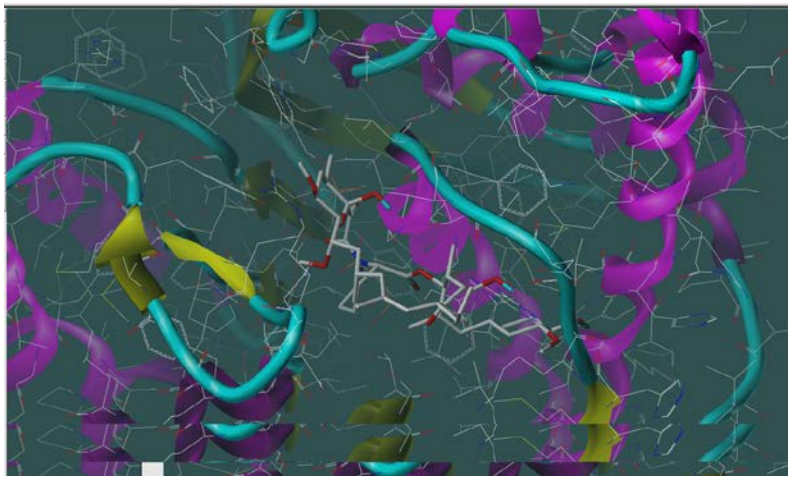

Figure 22. Tacrolimus, Docking score=-15.52

4.4 CONCLUSION

The computational approach gave us an insight into which drugs are likely to interfere with the pyridoxal kinase activity. This virtual approach was utilized in early drug discovery and in identification of probable targets. The concept was utilized since we have the crystal structure of the enzyme was available and the medications used for prediction of any possible drug interaction. All compounds were screened for fitness to target enzyme and the docking scores suggested few of the drugs as a good fit. The higher the docking score the better is the inhibitory effect and the compounds with lower docking score is not likely to be an inhibitor.

Tacrolimus is not likely to fit into pyridoxal kinase pocket due to its large molecular structure. In addition, direct complexation reaction with pyridoxal 5 phosphate is not likely, as the chemical structure of tacrolimus shows no free primary amine group; therefore the formation of Schiff base complex like isoniazid and other known pyridoxal kinase inhibitors is not feasible. Schiff base is formed by condensation of aldehyde or ketone with primary amines. Further testing for these compounds were included in the upcoming studies to further explain whether this spatial configuration fitness can or will translate to effect on activity or expression of pyridoxal kinase. Several drugs notably have a high docking score like pantoprazole (7.17) and trimethoprim (6.54). Compounds with high score were tested using human hepatocytes and the pyridoxal kinase expression was determined.

5.0 PYRIDOXAL KINASE EXPRESSION AND ACTIVITY

ABSTRACT:

Pyridoxal kinase is the enzyme that is responsible for activation of vitamin B6 vitamers into pyridoxal 5' phosphate (P5P), which is the ultimate active form of vitamin B6. Human hepatocyte cultures were used to study the effect of some drugs as well as some cytokines on the enzyme expression by measuring the mRNA expression using RT-PCR. Tacrolimus, pantoprazole, mycophenolic acid, IL-1, IL-2, and TNF-α were all evaluated for their effect on mRNA expression of pyridoxal kinase in cultured human hepatocytes. Both Tacrolimus(20 μg/L) and TNF-α inhibited the mRNA expression of pyridoxal kinase (p-value <0.0049). These results support the prediction that immunosuppressive drugs regimen based on tacrolimus and/or certain cytokines may affect the expression of pyridoxal kinase enzyme in liver.

The activity of pyridoxal kinase was evaluated in human liver extract and cultured human hepatocytes. The activity was assessed by adding the probe substrate pyridoxal and by consequently measuring the pyridoxal 5' phosphate metabolite. The acute effect of different tacrolimus concentrations on pyridoxal kinase activity in liver extract were compared with the known inhibitor 4-deoxypyridoxin. The chronic effect of different concentrations of tacrolimus and various cytokines on the activity of pyridoxal kinase in human hepatocytes cultures was

evaluated as well. The liver extract treatment with tacrolimus showed no effect on activity regardless of concentration. The hepatocyte treatment showed some inhibitory effect at ≥10µg/L tacrolimus concentrations and with some cytokines (IL-1β, IL-2, TNF-α). These results support the hypothesis of that tacrolimus (minimally) as well as some of the cytokines (significantly) may contribute to the reduced activity of pyridoxal kinase.

5.1 PYRIDOXAL KINASE EXPRESSION AND IN HUMAN HEPATOCYTES

5.1.1 Introduction

Pyridoxal kinase enzyme that is mainly responsible for the formation of pyridoxal 5'phosphate (P5P) was studied after treating human hepatocytes with different cytokines, various concentrations of the primary immunosuppressant (tacrolimus), pantoprazole and mycophenolic Acid (MPA), using the known inhibitor 4-deoxypyridine as control. The expression was evaluated by RT-PCR following the detailed protocol described later.

Human Hepatocytes are widely used in metabolism studies due to their reduced complexity and its relevance to the human liver function. However, the low expression levels of drug metabolizing enzymes and lack of continuous availability are among the disadvantage of this system. In addition, the lack of co-factor providing cells, such as Kupffer cells may render

these systems inefficient [112] The living system of hepatocytes, although relatively short lived, is convenient for the inhibition studies that will take 3-5 days in length.

5.1.2 Methods

Human Hepatocytes were received from Dr. Steven Strom Laboratory at the University of Pittsburgh. They were prepared from livers not suitable for transplantation. Hepatocytes were isolated by a three-step collagenase perfusion technique and plated on collagen coated 6 well plates. Cells were cultured in hepatocyte maintenance media (HMM) and maintained at 37 °C incubator throughout the experiment. Media were changed every 24 hours.

Hepatocytes were pre-treated with different concentrations of tacrolimus for 3 days that were within the therapeutic range (0, 2, 5, 10, 15, 20 μg/L). Additional plates were used for the following cytokines (IL-1, IL-2, TNF-α) at 10 ng/ml concentration that represent cytokine concentrations used in similar studies. These cytokines were selected as these three were reported in the literature for their effect on the metabolizing enzymes and transporters [106]. Two plates were used for pantoprazole and mycophenolic acid (MPA) at concentration of 4 μg/ml, which is close to the maximum concentration observed in plasma in patients on these medications. These two medications had the highest docking scores. On day 4, media were removed from the plates and cells were washed twice in ice cold phosphate buffered saline (PBS). Total RNA was extracted using 1 ml of Trizol according to manufacturer's protocol. The scraped samples were kept at -80 °C until further RT-PCR analysis. Purified total RNA was reverse transcribed at using appropriate primers for pyridoxal kinase in addition to house-keeping gene (Cyclophillin), accordingly.

The following process was followed for primer design, using “Nucleotide” library in” PubMed”, the search was conducted by encoded gene’s name “PDXK” with specifying the species as humans (Homo Sapiens. The selected mRNA was chosen and a sequence was picked. The entire gene sequence was copied and utilizing “BLAST” program to identify sequence similarities. The region that has the longest distance between two adjacent exons was chosen. The new sequence was pasted into http://frodo.wi.wi.mit.edu/ to get the following primers:

Homo sapiens (PDXK) 1

LEFT PRIMER ctccatacagagccacgtca
RIGHT PRIMER acagagttcaccgcgtcaat

Homo sapiens (PDXK) 2

LEFT PRIMER catacagagccacgtcatcc
RIGHT PRIMER cctgtgtggtttgaaaactgg

A mixture of 50 µL of right and left primers were used in PCR reaction

The samples were prepared according to standard protocol, trizol was added to tissues and homogenized in test tube, 200 µL chloroform was added into 1 ml of homogenate in an Eppendorf tube, total RNA extraction was continued according to protocol. (Appendix D). The following step aimed to reverse transcription, 11 µL of RNA extracted from previous step was added to 1µL of primer and dNTPs. After running samples in PCR for 5 minutes, buffer, DTT and reverse transcriptase enzyme were added to samples and ran in PCR (Two steps reaction). This process yielded the cDNAs. Two µL of cDNA resulted from previous step was mixed with SYBR green dye with buffer and primers specific to PDXK gene. Finally RT-PCR was run

according to the protocol in the manual. The following instruments were used: Eppendorf master cycler gradient 5331 (PCR), Eppendorf 5424 microcentrifuge with knobs and Applied Bio systems 7900HT Fast Real Time PCR System.

5.1.3 Results

The mRNA expression of enzyme was compared to house-keeping gene of choice. In our experiment PDXK was compared to Cyclophilin as the house keeping gene and the fold change was calculated. The method used for calculation is called (Delta Delta Ct Method) from manufacturer protocol. The following equations were used where X is the tested gene, HK is the house keeping gene, and C is the cycle number

$\Delta\Delta Ct = \Delta Ct\ (X_{treat} - HK_{treat}) - \Delta ct\ (X_{control} - HK_{control})$, the fold change = $2^{(-\Delta\Delta Ct)}$

All treatments when compared together with single factor ANOVA for the effect on pyridoxal kinase mRNA expression in hepatocytes. There was no significant difference between groups (P-value =0.132) as shown in Figure 23.

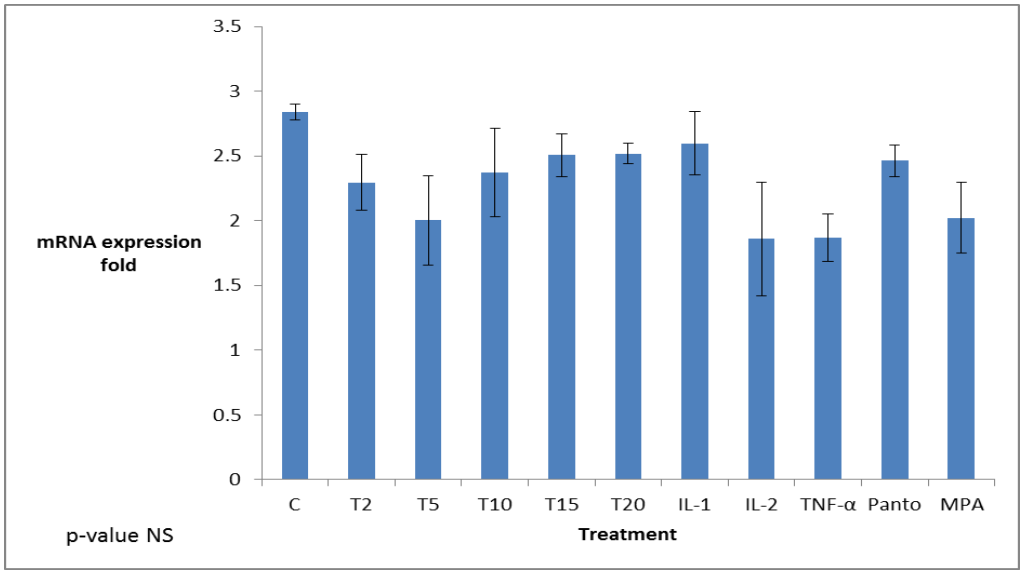

Figure 23. Pyridoxal kinase mRNA expression in hepatocytes with different treatments, C: control, T: tacrolimus (2, 5, 10, 20 μg/L), IL-1: interleukin 1, IL-2: interleukin 2, TNF-α: tissue necrosis factor α, Panto: Pantoprazole, MPA: mycophenolic acid

The post-hoc Bonferroni correction analysis uncovers that TNF-α and tacrolimus at concentration 20 μg/L are statistically significantly different from the control in mRNA expression of pyridoxal kinase. The expression study suggested that the rest of the drugs and cytokines do not have a direct inhibitory effect on the pyridoxal kinase enzyme. The statistical analysis is shown on Table 24.

Table 24. ANOVA post hoc analysis pyridoxal kinase mRNA expression in hepatocytes treated with drugs and cytokines

Treatment	P-value Bonferroni Correction (<0.0049)
Tacrolimus 2 µg/L	0.013718
Tacrolimus 5 µg/L	0.015058
Tacrolimus 10 µg/L	0.118384
Tacrolimus 15 µg/L	0.070274
Tacrolimus 20 µg/L	**0.00357**
IL-1	0.305184
IL-2	0.02129
TNF-α	**0.00019**
Pantoprazole	0.008077
MPA	0.005301

5.1.4 Conclusion

Most of the treatments were not statistically different than the control in terms of inhibiting the mRNA expression of pyridoxal kinase in human hepatocytes. Only the post hoc comparison suggested that Tacrolimus at 20µg/L and TNF-α might have an effect on pyridoxal kinase expression.

The computational docking exercise revealed that few medications may interact with pyridoxal kinase. Of the various drugs tested, pantoprazole scored the highest which led to further investigation. The RT-PCR experiment showed that only the highest concentration of tacrolimus tested had an effect on mRNA expression of pyridoxal kinase while other medications including lower tacrolimus concentrations, pantoprazole or mycophenolic acid had no effect on mRNA expression of pyridoxal kinase. Neither of these two agents was used in all patients who developed vitamin B6 deficiency. In addition, the cytokine (TNF-α) reduced the mRNA expression of pyridoxal kinase in hepatocytes, which suggested that this cytokine may also contribute to the observation in patients. Elevated levels of TNF-α were reported in small bowel transplant patients who developed vitamin B6 deficiency, which is consistent with current results.

Elevated serum levels of IL-2, IL-6, and TNF-α had been reported in the early session (about 40 days) following small bowel. (113) Also IL-6 mRNA expression in the transplanted small bowel tissues was up regulated significantly early in the reperfusion phase during transplantation [114]. These results suggested an important role for certain cytokines in small bowel transplantation, but the effect on pyridoxal kinase activity or expression had not been reported before.

Limitation: the expression study was solely focused on the mRNA pyridoxal kinase which is highly specific and sensitive utilizing the quantitative RT-PCR. However, the pyridoxal kinase protein expression was not measured or evaluated and that can be hard to interpret. A western blot targeting pyridoxal kinase will confirm that the expression is affected accurately. RT-PCR used has an advantage of being feasible, reproducible and highly sensitive.

5.2 PYRIDOXAL KINASE ACTIVITY IN HUMAN LIVER HOMOGENATE AND HUMAN HEPATOCYTE CULTURES

5.2.1 Introduction

Pyridoxal kinase is a cytosolic enzyme that is abundant in liver tissue. It is not clear how the vitamin B6 deficiency develops following organ transplantation. The approach used here is to evaluate the impact of all variables like immunosuppressive medications and elevated cytokines relevant to small bowel transplantation on the activity of pyridoxal kinase. The acute effect of the primary immunosuppressive drug (tacrolimus) was evaluated in human liver homogenate over range of concentrations. The chronic effect was evaluated using human hepatocytes cultures over a range of concentration of tacrolimus and with cytokines over a period of four days.

5.2.2 Methods

The effect of acute treatment with tacrolimus on pyridoxal kinase activity in human liver homogenate was evaluated using pyridoxal as probe substrate. Pyridoxal kinase activity - expressed as pyridoxal 5' phosphate conc. (μg/L)/mg protein.1hr- was measured at different tacrolimus concentration that included the therapeutic concentrations (0, 2, 5, 10, 15, 20 μg/ml) in the presence of 20 μM Pyridoxal in (100 μg protein) liver cell homogenate, The inhibitor 4-

deoxypyridoxine (10 μM) served as a control. All these treatment were done in triplicates and the Mean ± SE were compared to control. Pyridoxal 5' phosphate was measured by HPLC.

The effect of chronic treatment with tacrolimus on pyridoxal kinase activity in human hepatocytes was evaluated using pyridoxal as probe substrate. Hepatocytes were pre-treated with different concentrations of tacrolimus that included the therapeutic concentrations (0, 2, 5, 10, 15, 20 μg/L), for three days, on day 4 the hepatocytes were treated with the tacrolimus in the presence of 20 μM Pyridoxal, the probe substrate for pyridoxal kinase. The inhibitor 4-deoxypyridoxine (10 μM) served as a positive control. Pyridoxal kinase activity was expressed as pyridoxal 5' phosphate conc. (μg/L)/10^6cells. All treatments were performed in 6-well plates and were compared to control.

The effect of cytokines on pyridoxal kinase activity in human hepatocytes was evaluated in similarly like above. Human liver hepatocytes were treated with 50 pg/ml cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α) for 3 days; 4-deoxypyridine was used as control inhibitor. On day 4 all hepatocytes were treated with the substrate (Pyridoxal) and incubated as previously described. Pyridoxal kinase activity was measured by measuring P5P formed with different treatments. All treatments were done in 6-well plates and the mean± SE were compared to control.

5.2.3 Results

Calibration Curve of Pyridoxal 5` Phosphate in Buffer was constructed using HPLC. The curve showed linearity over the concentration range tested with an R^2 =0.999 as shown on Figure 24.

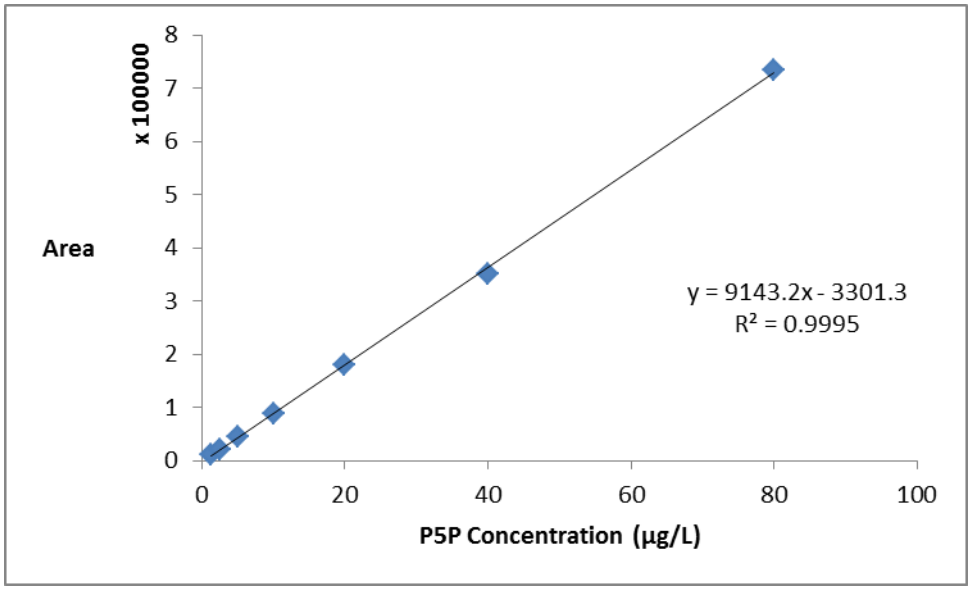

Figure 24. Pyridoxal 5'phosphate calibration curve in buffer

The formation of pyridoxal 5` phosphate in human liver homogenates was not affected at any concentration of tacrolimus. The pyridoxal kinase is a cytosolic enzyme that can be degraded by the proteases and peptidases upon liver homogenate preparation; however the buffer system included inhibitors for these enzymes. The tissue levels of tacrolimus at the human liver are undefined so we tested multiple concentrations of tacrolimus. In addition, a high concentration of the substrate was justified in order to make the substrate available to the enzyme and to have detectable amounts of the metabolite within one hour. The pyridoxal kinase activity measured by P5P concentration/mg protein in 1 hour treatment was compared to the control and to the positive inhibitor 4-doxypyridoxine treatment. The results after one hour treatment showed no difference from the control as shown in Figure 25.

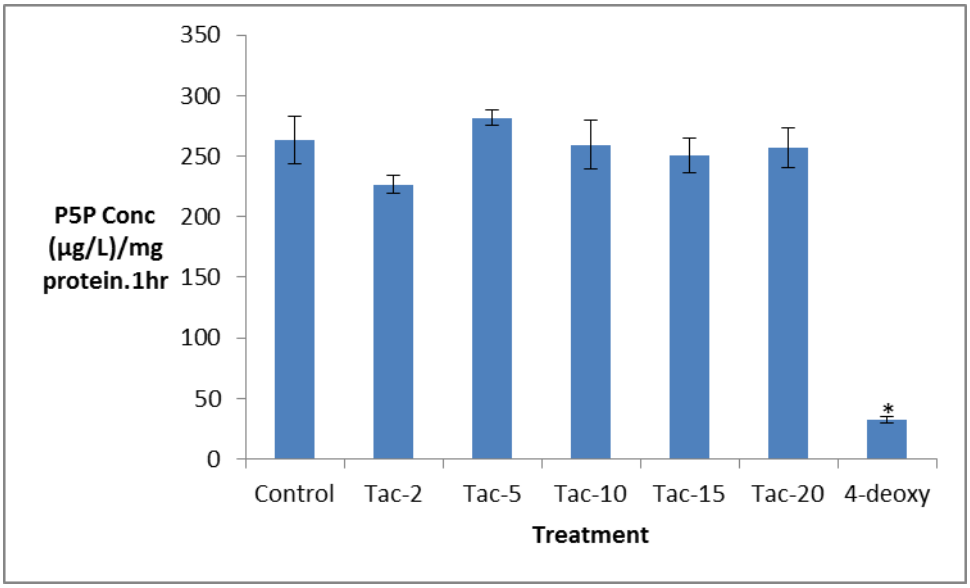

Figure 25. Pyridoxal 5' phosphate level in human liver homogenate after treating with different concentrations of tacrolimus (n=3)

The comparison between different P5P levels generated in presence of different concentrations of tacrolimus suggested that only tacrolimus can potentially inhibit pyridoxal kinase activity at higher concentrations. However the maximal inhibition did not exceed 20% even with the highest concentration (Figure 26).

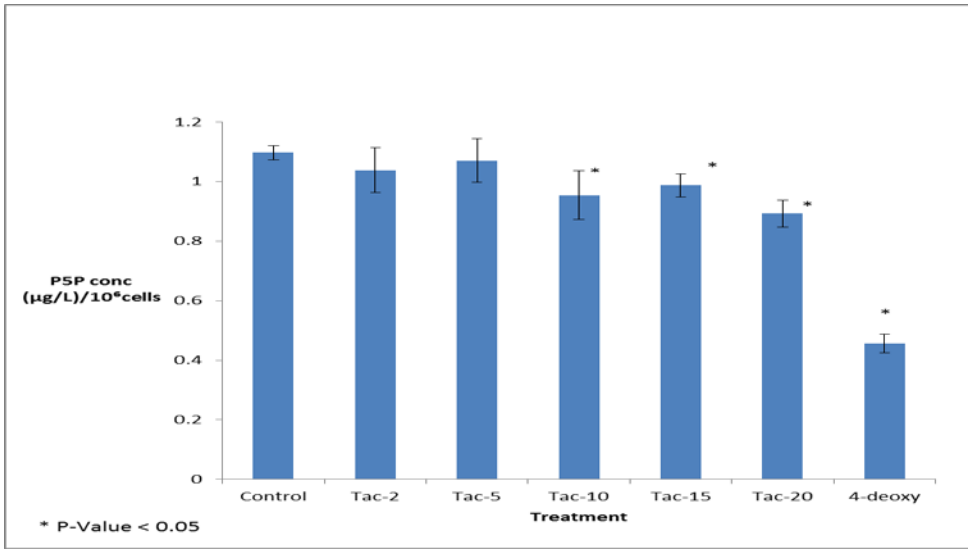

Figure 26. Pyridoxal 5' phosphate level in human hepatocytes after treating with different concentrations of tacrolimus (n=6)

The effect of cytokines on pyridoxal kinase activity was noted with IL-1β, IL-2, and TNF-α. The other cytokines did not have an effect on pyridoxal kinase activity in human hepatocytes system. However, all these cytokines were applied in a 50 pg/ml median concentration. The results are shown on Figure 27.

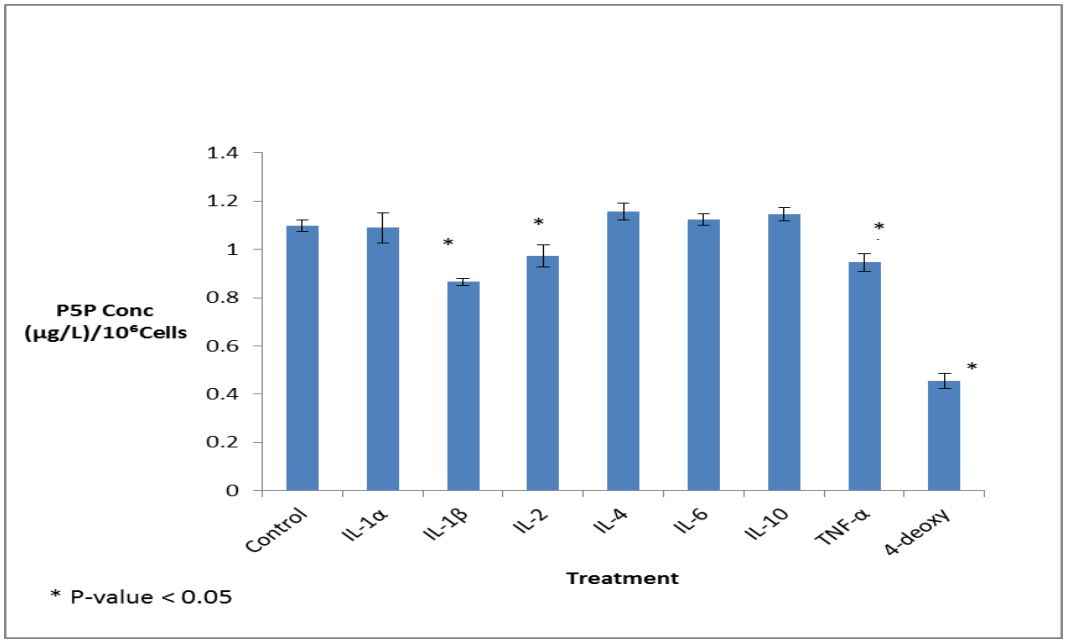

Figure 27. Pyridoxal 5' phosphate level in hepatocytes after treating with different cytokines (n=6)

Limitations: the activity studies in hepatocytes resemble closely the living system; however is not exactly the same. The time of incubation with the substrate can be a variable, however an hour of incubation with the substrate can be extended in order to get more of the formation reaction might take place. The one hour incubation was adopted from other published experiments.

The activity studies in the liver homogenate were utilized for the acute effect of different agents on pyridoxal kinase. The pyridoxal kinase is a cytosolic enzyme that can be degraded by the proteases upon homogenization or damaged by oxidation. However, the homogenizing buffer included phenylmethylsulfonyl fluoride as a serine protease inhibitor and butylated hydroxytoluene as antioxidant that terminate auto-oxidation. In addition, using a standard sample preparation protocol might help standardization of the procedure to minimize variation.

5.2.4 Conclusion

The primary hepatocyte cultures provided a living system that resembles closely the cellular system that include all cellular components being intact and the drugs would exert their action in a similar way like *in vivo*. However, the inhibition of pyridoxal kinase activity by tacrolimus in hepatocyte was marginal but yet statistically significant. The results showed that tacrolimus did not significantly affect the activity of pyridoxal kinase in human liver extract regardless of the concentration. The effect was comparable to the control while it showed a significantly different activity with the true positive control (4-deoxypyridoxine), which is a known inhibitor to pyridoxal kinase. However, tacrolimus effect on primary cultured hepatocytes was statistically significant at higher concentrations of tacrolimus (> 10 μg/L).

Treating hepatocytes with IL-1β, IL-2, and TNF-α resulted in lower pyridoxal kinase activity, while other cytokines did not. These specific cytokines were reported in small bowel transplantation but their effect on pyridoxal kinase enzyme was not yet investigated. All these cytokines IL-1β, IL-2, TNF-α were reported in different literature for being elevated following small bowel transplantation and would sustain their higher than control levels for few months to a year following transplantation [113, 114]. The effect of different cytokines on pyridoxal kinase activity is a new finding that need to be further investigated.

High concentrations of tacrolimus and selected cytokines appear to decrease the activity of pyridoxal kinase. This study provided support for the prediction that tacrolimus and cytokine (TNF-α) may contribute to altered pyridoxal kinase activity in patients.

5.3 EFFECT OF TACROLIMUS ON PYRIDOXAL 5'PHOSPHATE BINDING TO PLASMA PROTEIN

5.3.1 Introduction

Tacrolimus is the primary immunosuppressive agent used in small bowel transplantation. It has a very well characterized distribution in the body and binds to protein and red blood cells. On the other hand, pyridoxal 5' phosphate (P5P) also has similar distribution profile which suggests another possibility for drug interaction. An *in vitro* protein binding study was carried out with different tacrolimus concentrations and the free P5P fraction was measured and compared to control. The results of the *in vitro* experiment did not suggest an important interaction.

Tacrolimus is distributed in blood and plasma in a very similar fashion like the pyridoxal 5` phosphate. Both bind to red blood cells and plasma proteins. The drug interaction in terms of binding was explored by mixing pyridoxal 5' phosphate in plasma at different concentrations of tacrolimus and separating the bound P5P from the unbound P5P using ultrafiltration device, then measure the free pyridoxal 5` phosphate by HPLC.

5.3.2 Methods

Human plasma was incubated with various concentrations of tacrolimus (0, 2, 5, 10, 15, 20 μg/L) and 20 μg/L pyridoxal 5' phosphate (P5P), then ultra-centrifuged using the Millipore ultrafiltration device. The supernatant was collected and the free P5P concentrations were

measured by HPLC. The calibration curve was constructed using HLPC conditions similar to what we used with buffer samples with an additional orthophosphoric acid pre-treatment step for sample preparation to precipitate the plasma proteins. The rest of the procedure was exactly the same as described earlier.

5.3.3 Results

The P5P calibration curve in plasma was linear across the concentration range used with R^2=0.999 that indicated an excellent linearity. The calibration curve is shown in Figure 28. The results of the binding study showed minimal to no effect of tacrolimus on binding pyridoxal 5` phosphate to plasma protein. This might be explained by the higher affinity of the smaller molecule to bind to albumin than that of the larger tacrolimus. The results expressed as P5P percent bound in plasma are shown in Figure 29.

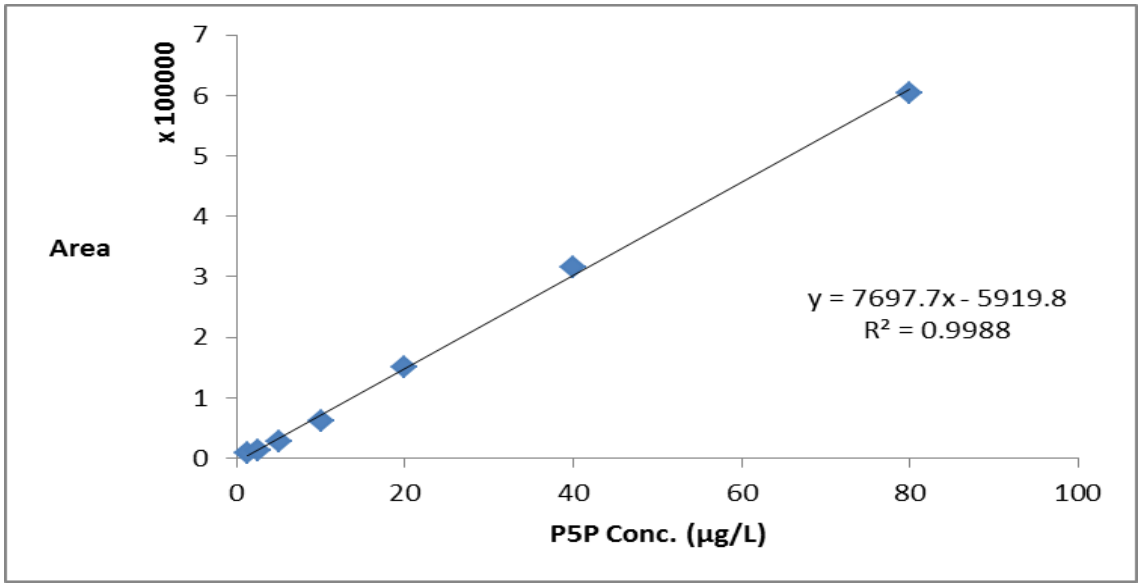

Figure 28. Pyridoxal 5' phosphate calibration curve in plasma

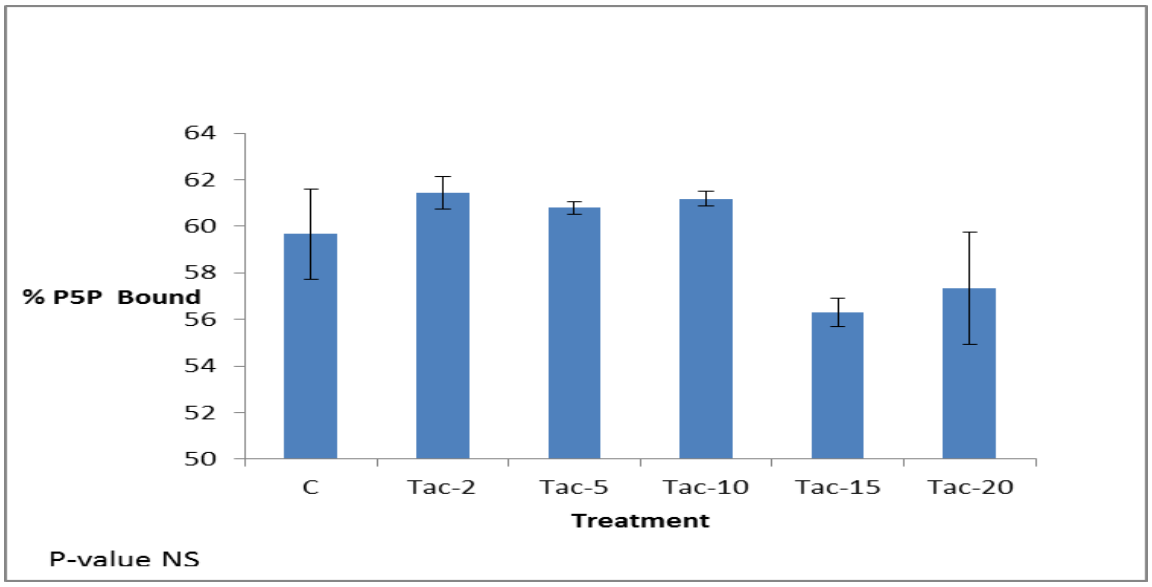

Figure 29. Percent pyridoxal 5' phosphate bound in plasma treated with different tacrolimus concentrations (n=6)

Limitations: The plasma levels of free P5P along with albumin might provide a more accurate answer. The experiment suggested unlikely interaction at albumin level.

5.4 CONCLUSIONS

To support the hypothesis of the effect of drugs and/or cytokines on pyridoxal kinase activity and expression several *in vitro* evaluation tests were initiated utilizing human liver extract and human cultured hepatocytes. The activity of pyridoxal kinase was measured by the metabolic product of the probe substrate pyridoxal. The product pyridoxal 5` phosphate is the major metabolite that was measured in buffer and plasma using the adopted HPLC technique.

The computational approach screened all the medications commonly used in transplant patients. Tacrolimus scored low and a direct interference with pyridoxal enzyme is not likely. Other medications like pantoprazole and mycophenolic acid had a high docking score and was evaluated *in vitro* for their potential interaction role.

Pyridoxal kinase activity in human liver extract and human cultured hepatocytes was evaluated in the presence of different concentration of tacrolimus and several selected cytokines. Tacrolimus did not affect the metabolism of pyridoxal in human liver extract and showed some inhibition only at high concentration ≥10μg/L only in human hepatocytes. The following cytokines (IL-1β, IL-2, and TNF-α) had an inhibitory effect on pyridoxal kinase activity in hepatocytes.

The *in vitro* testing utilized human liver extract for acute effect of different medications (tacrolimus, pantoprazole, and mycophenolic acid) on the activity of pyridoxal kinase. The result suggested no effect of tested drugs on pyridoxal kinase activity. The use of human hepatocytes cultures for chronic effect of these drugs and cytokines were evaluated for activity and expression. Only tacrolimus at higher concentrations 10-20μg/L and IL-1β, IL-2 and TNF-α showed an effect on inhibiting pyridoxal kinase activity in hepatocytes. The mRNA expression of pyridoxal kinase in hepatocytes were only significant with TNF-α and the highest tacrolimus concentration used (20μg/L).The plasma protein binding study with tacrolimus showed a no effect with all used tacrolimus concentrations. These findings support a probable role of cytokines in altering pyridoxal kinase activity and also that might be augmented by higher concentrations of tacrolimus.

6.0 PYRIDOXAL KINASE ACTIVITY AND EXPRESSION IN TRANSPLANTATION- OBSERVATION IN AN ANIMAL MODEL

Abstract:

Vitamin B6 deficiency appears to be present in all transplant patient population. A transplant model that does not involve an organ that participates in absorption, distribution, metabolism or excretion of medication provides an opportunity to test the impact of the effect of transplantation process and associated inflammation on vitamin B6 status. The composite allogenic transplant animal model where the hind limb was transplanted provided an excellent model to study the effect of transplantation of a remote organ that is highly immunogenic on the functional capacity of other tissues and organs in the body. Iso graft transplantation where the rejection should be absent, allogenic transplantation where the rejection is expected to happen with all its consequences and an allogenic transplantation where the immunosuppressive regimen is used, where rejection is prevented, was used in this study. It was possible to study the effect of transplantation, the acute rejection, and the effect of the immunosuppressive medication using this approach. The harvested livers extract were evaluated for pyridoxal kinase activity using pyridoxal as probe substrate. The total protein amounts in their liver extract were significantly less in all transplant animal groups and were the least in allogenic transplant group. The pyridoxal kinase activity and expression were less in the allogenic transplant group when

compared with all other groups. This study supports that transplantation surgery as well as the rejection process both contribute to decreased expression of pyridoxal kinase in the liver. The conclusion is that every time there is acute rejection the levels of inflammatory markers like cytokines and alkaline phosphatase will be increased and that might lead to increased vitamin B6 degradation and deficiency or at least a higher need for vitamin B6 in transplant patients.

6.1 INTRODUCTION

Composite tissue allotransplantation (CTA) has drawn a lot of attention recently as an experimental model that target partial and complete faces, hands, forearms, arms, abdominal walls, bones, and bone-joint complexes. The whole process of transplantation, immunomodulation, rejection consequences, and therapeutic options are quite similar to other solid organ transplantation. Composite allotransplantation is known for its high immunogenic rejection response which might have effects on other non-transplanted organs in the body [115]. Unlike other allografts, the transplanted tissues in CTA are histologically heterogeneous, includes many tissue types (skin, muscle, bone, lymph nodes, nerves and tendons), and thus present variable immunogenicity of transplanted tissues to the host. This mandates intense and life-long immunosuppressive strategies [116]. Our utilization of this model is because of the augmented immunological response that might give us an insight for better understanding of the effect of transplantation and acute rejection on different non-transplanted organs like liver [117]. The mechanisms and mediators of inflammation in potential models for skin rejection in CTA had been partially understood. Chemokines or the cytokines with chemotactic activity for

leukocytes are reported to play an important role in the acute rejection processes and further their corresponding receptors expression up-regulation in various solid organ transplants as well as CTA models [118].

We hypothesized that transplantation as process with its inflammatory cascade as well as the immune response may affect the functional capacity of the liver where most of the metabolism in the body took place. Pyridoxal kinase (PK) is mainly a hepatic enzyme that is responsible for the metabolism of vitamin B6 to its active precursor pyridoxal 5` phosphate. The animal model included an isograft rat group where same genetically matched animals were donor and recipient of the hind limb. The model also provided allogenic rat groups, where the donor is mismatched with the recipient and so that the rejection occurs. Half of this group received antirejection regimen with tacrolimus and was not expected to be rejected. All these groups will be also compared to healthy non-transplanted control group of rats. The liver was the target organ of interest, where the activity as well as the expression of pyridoxal kinase was evaluated.

6.2 METHODS

6.2.1 Animals

The study in rats was approved by Institutional Animal Care and Use Committee at University of Pittsburgh, Approval # 858. The use of rats in this experiment was justified by these animals being the smallest that may allow the surgeon to carry out the hind limb

transplantation. All rats were weighed 200–250 g and all animals were kept in individual mesh cages in a 12 hour day/night cycle.

All surgeries were performed under sterile conditions using sterile instruments. Anesthesia was provided using sodium pentobarbital (50mg/kg, i.p). Surgical time was between 3 to 5 hours. For the extended anesthesia Nembutal (10mg/kg) i.p booster doses was used. The depth of the anesthesia was assessed by movement of the animal and the frequency of respiration and by administering the toe pinch withdraw reflex test to the animal. The donor animal's hind-limb and groin were shaved, and the animal was placed in a supine decubitus position. The skin was prepped with betadine or chlorhexidine surgical prep, followed by 70% alcohol. An inguinal skin incision was made, exposing the epigastric vessels, which were cauterized. The femoral sheath was identified and dissected out, separating the nerve, artery and vein components. These were divided sharply ensuring adequate length for later anastomosis. Attention was then turned to the postero-lateral aspect of the limb, extending the skin incision, transecting the superficial muscle layer, and identifying and isolating the sciatic nerve. The nerve was then sharply transected. The remaining muscle groups were then transected to completely expose the mid-portion of the femur. Using a sharp bone cutter, a 90 degree osteotomy was performed, and the allograft was freed from the remainder of the donor animal. The procedure was repeated to harvest the contralateral limb to minimize the number of donor animals required. Allograft donor animals were sacrificed following procurement of the allograft. The recipient animal were shaved and prepped in a similar fashion under sterile conditions. Implantation of the allograft proceeded with osteosynthesis using a 22-gauge needle as an intermedullary rod. Major ventral muscle groups were reapproximated with 4-0 suture.

The femoral vein and then femoral artery were anastomosed using standard micro vascular technique and 10-0 suture.

Once the allograft was reperfused, the dorsal sciatic nerve was approximated in a similar fashion with 10-0 suture and the dorsal muscle groups were sutured together with 4-0 suture. Skin closure was performed using 4-0 silk in a running fashion. Skin sutures were absorbable and therefore do not require removal, minimizing the potential trauma to the recipient. All wounds were closed in an interrupted fashion in case the suture breaks. Once surgery was completed, the animals were then placed under a heat lamp until they were awake. Upon awakening the animals were transferred to the post-operative facility where they received food and water ad libitum.

6.2.2 Study design

The Lewis rats were assigned into four groups according to limb transplant allocation

- Isograft group,(Auto)
- Allograft group, (Allo)
- Allograft treated with tacrolimus group, (Allo-Tac)
- Control group (C)

The tacrolimus treated group received intraperitoneal injection of 0.5 mg/kg/day. Animals were monitored for normal behavior and physiological function to ascertain their recovery from anesthesia, and assessment of pain/distress. The animals were warmed by circulating water heating pads or heat lamp and were monitored until the normal reflex was

regained. Pain was alleviated with buprenorphine (0.1 mg/kg Q12 hours) for 3 days. The animals were allowed to ambulate in the post-operative period. Limbs were evaluated daily for evidence of rejection or dehiscence of the suture. This involved observing the limb for occurrence of edema, erythema, epidermolysis, desquamation and mummification. The animals were monitored twice daily until sacrifice. When acute rejection occurred, respective animals were euthanized using an overdose of sodium pentobarbital (i.p, 100 mg/kg). We considered weight loss >20% of age matched controls to be significant. All animals were observed daily for infection and weight loss of more than 20%.

We expected that the group receiving no immunosuppression after transplantation will show evidence of rejection by 4-7 days based on past experience. On day 5 post transplantation, animals were sacrificed and livers were collected and stored at – 80 °C until analysis for pyridoxal kinase expression.

6.2.3 Liver homogenate preparation

The livers were collected from each rat in each group on day 5 post operation. The livers were frozen in liquid nitrogen and kept at -80 °C for expression analysis. The liver homogenates were prepared as described in Appendix A. Pyridoxal kinase enzyme is a cytosolic enzyme and so the supernatant was used after the centrifugation at 10,000 g for 20 minutes.

6.2.4 Total protein assay

Total protein assay was performed utilizing the Lowry's method. The calibration curve was constructed using bovine serum albumin (BSA) ranging in concentration from 0.025 – 0.4 mg/ml. The UV absorption was measured using colorimetry at 750 nm.

6.2.5 RT-PCR

The pyridoxal kinase protein expression in different transplanted rat groups was measured by RT-PCR using actin as housekeeping gene. Purified total RNA was reverse transcribed using appropriate pyridoxal kinase primers in addition to house-keeping genes accordingly.

The primer design was performed according to protocol, using "Nucleotide" library in" PubMed", the search was conducted by encoded gene's name "PDXK" with specifying the species as rats (Rattus norvegicus (PDXK). The selected mRNA was chosen and a sequence was picked. The entire gene sequence was copied and utilizing "BLAST" program to identify sequence similarities. The region that has the longest distance between two adjacent exons was chosen. The new sequence was pasted into http://frodo.wi.wi.mit.edu/ to get the following primers:

Rattus norvegicus (PDXK):

LEFT PRIMER ctctccatccagagccatgt

RIGHT PRIMER cacagagttcacggcatcaa

The sample preparation was carried out according to standard protocol, Trizol was added to tissues and tissues were homogenized in test tube, 200 μL of chloroform was added into 1 ml of homogenate in an Eppendorf tube, and total RNA extraction was performed according to protocol. The reverse transcription reaction was carried out according to protocol, eleven μL of RNA extracted from previous step was added to 1μL of primer and dNTPs. After running samples in PCR for 5 minutes, buffer, DTT and reverse transcriptase enzyme to samples were added and ran it in PCR (Two steps reaction). These steps yielded the cDNAs. Two μL of cDNA resulted from previous step was mixed with SYBR green dye with buffer and primers specific to PDXK gene. Finally RT-PCR was run according to the protocol. The following instruments were used: Eppendorf master cycler gradient 5331 (PCR), Eppendorf 5424 microcentrifuge with knobs and Applied Bio systems 7900HT Fast Real Time PCR System. Decrease expression: The RT-PCR results in measuring the mRNA expression compared to house-keeping gene of choice (Actin) will show if there will be a reduction in amount of enzyme protein expressed. The method used for calculation is called Delta Delta Ct Method from manufacturer as previously described.

Pyridoxal kinase activity in the rat liver extract was also carried out as previously described. The concentration of P5P was determined after 1 hour reaction, which will be corresponding to the amount of protein needed for the reaction. The enzyme activity is defined as the concentration of P5P/mg protein in 1 hour at 37 °C.

6.3 RESULTS AND DISCUSSION

The total protein assay was linear over the range of the concentration used with R^2 =0.999. Figure 30 All animal groups underwent surgery showed a significantly lower total protein content when compared with the animals in the control group. However, the total protein content of both the allograft group and the tacrolimus treated allograft group were significantly lower than the isograft graft group. The underlying reason for this finding needs further investigation.

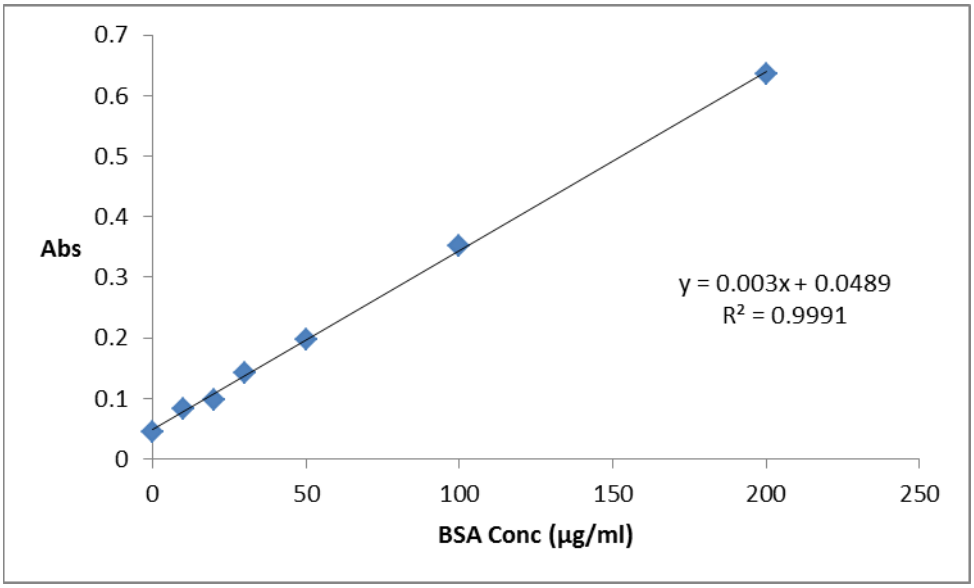

Figure 30. Total protein calibration curve

All Transplant rat groups total protein were compared using single factor ANOVA where all groups were significantly different from each other (p-value <0.05). All these liver extracts total protein content were corrected to liver weight. The post hoc bonferoni correction with a p-value =0.0125 for the all subgroup analysis. The post Hoc analysis confirmed a significant

difference between allogenic transplant groups and the control. Both allogenic transplant groups (Allogenic group and allogenic receiving tacrolimus group) were not different in total protein amount in the liver. The results are shown in Figure 31.

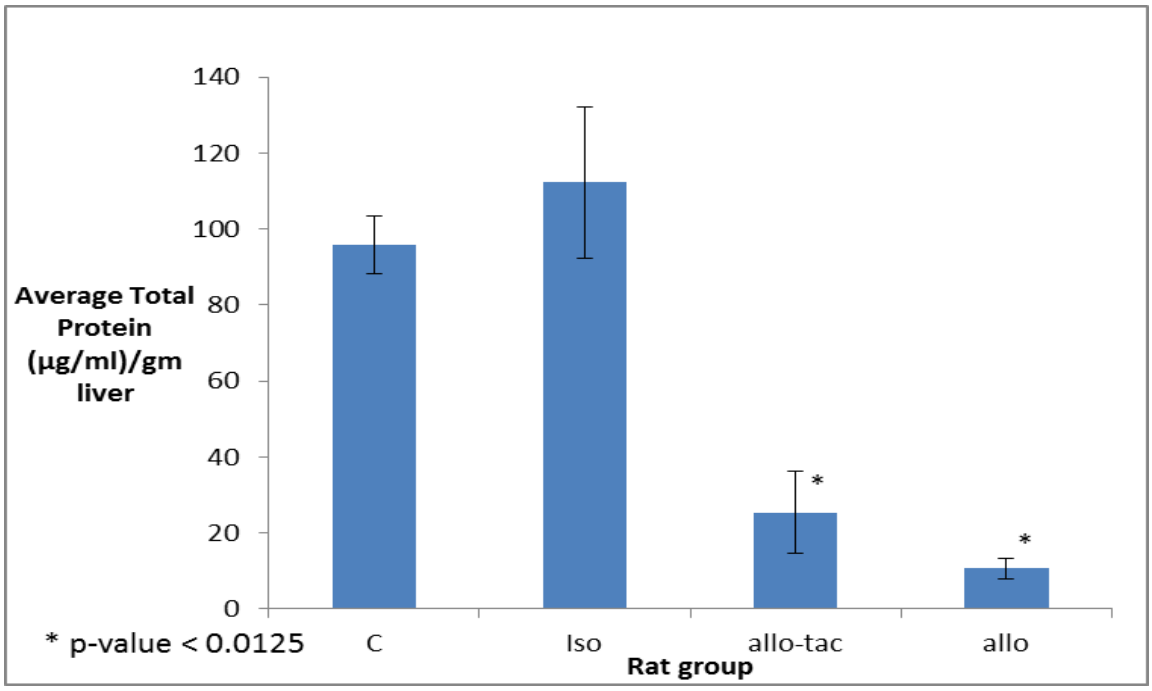

Figure 31. Total protein content in different limb transplant rat groups (n=5), C: control, Iso: isograft, allo-tac: tacrolimus treated allogenic, allo: allogenic

The mRNA expression in the livers of different rat groups was compared to controls and to each other. ANOVA comparison between groups was significant with p-value <0.05. The post Hoc analysis with Bonferroni correction (α/4) the allogenic rat groups were significantly lower than the control group. P-values were <0.0125 for allogenic group and for allogenic group treated with tacrolimus.

The lowest expression of pyridoxal kinase mRNA was in the allogenic transplant rat, which was significantly lower than the control group (p-value<0.0125). The other allogenic transplant group that received tacrolimus was also statistically significantly lower than control. The isograft group was not different from the control (p-value=0.22) .The effect on hepatic pyridoxal kinase following a different non-liver transplantation can be explained by the cascade of events that accompanied the rejection or the immunomodulation by elevated inflammatory markers. The results are shown in Figure 32.

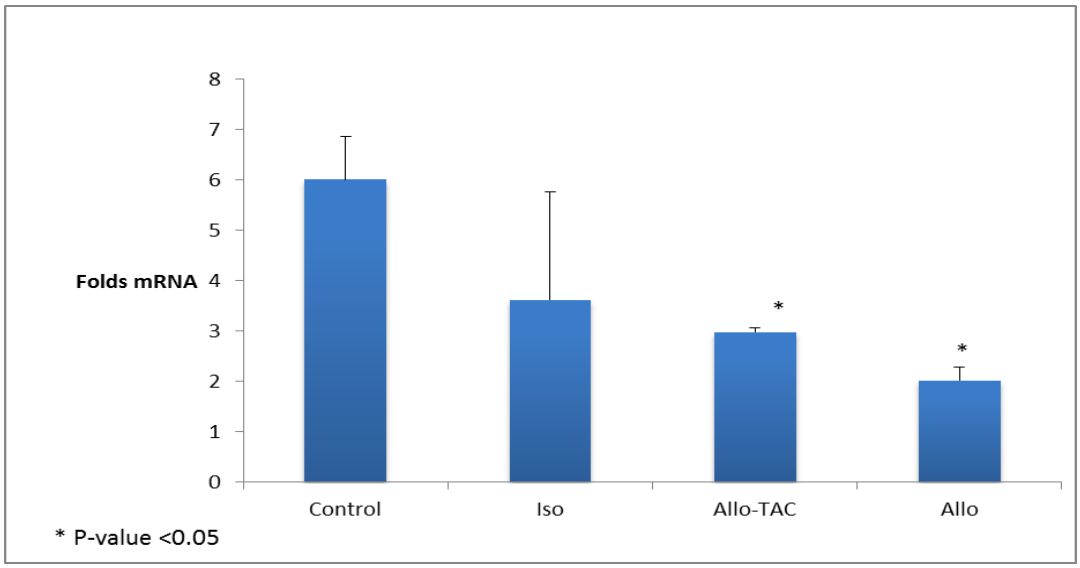

Figure 32. Pyridoxal kinase mRNA expression in different limb transplant rat groups (n=5), C: control, Iso: isograft, allo-tac: tacrolimus treated allogenic, allo: allogenic

The overall total protein content in liver in allogenic group without tacrolimus was also statistically significant compared to control and that might indicate an effect of the transplantation on a distant organ like the liver function despite the fact it is not the transplanted organ and consequently affect the different enzymes expression and function in the body. The

mechanisms so far are not well identified, but can be attributed to elevated levels of cytokines status post transplantation that might have an effect on liver function and other metabolizing enzymes like pyridoxal kinase. Also, the lower total liver protein might be used as an early predictor of rejection or allograft failure , serum albumin was suggested as an independent predictor of poor outcomes in kidney transplant recipients however the clinical adoption of such an initiative need further support and evidence [119].

The pyridoxal kinase activity measured by the metabolically active form pyridoxal 5` phosphate concentration with rat liver extract after incubation for an hour. The measured concentration was normalized to the total protein content and was significantly less in the allogenic transplant group when compared to all other groups. The activity was notably lower than that of the control in the allogenic transplant rat group while there was no statistically significant difference in the activity between any other groups. The results are shown in Figure 33.

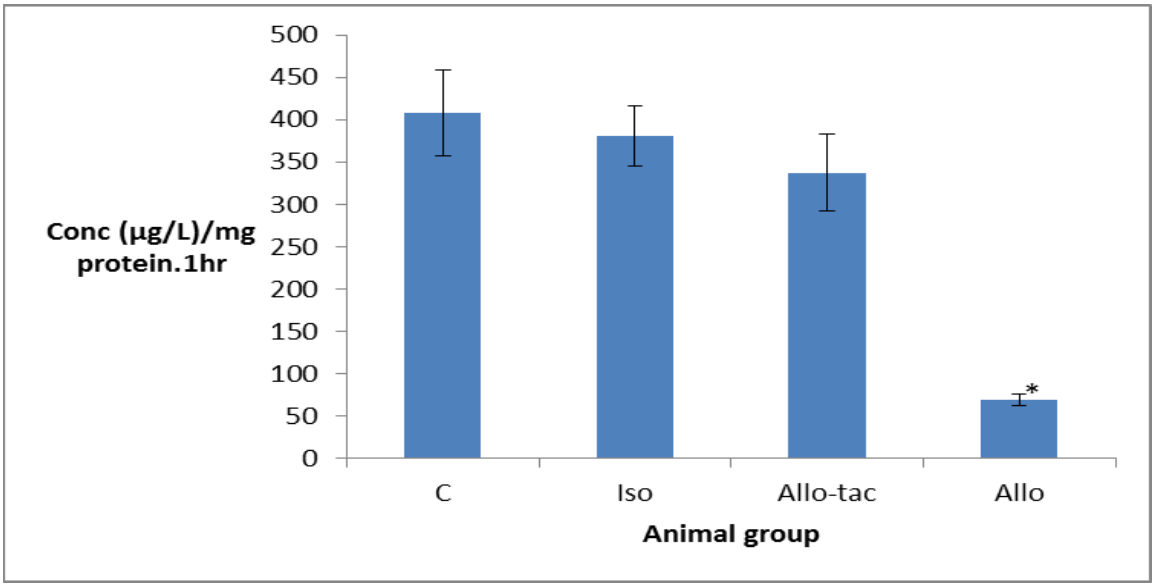

Figure 33. Average pyridoxal 5'phosphate concentration in different rat transplant livers extracts (n=5), C: control, Iso: isograft, allo-tac: tacrolimus treated allogenic, allo: allogenic

Limitations: composite tissue allogenic transplantation provides an experimental model to study the effect of transplantation on other organs. The differences between CTA and small bowel transplantation is not known or even evaluated. However, it is novel and innovative to study the non-transplanted organ for it's the global effect of inflammation/ transplantation on metabolic capacity of an organ like liver. The sample size was conveniently small rather adequate for the comparison purposes. Cytokines profiling in tissues and plasma might further explain these effects of the liver and other organs as well.

6.4 CONCLUSIONS

Transplantation is a process where the transplanted organ is subjected to inflammatory processes like ischemia/ reperfusion and rejection, both of which involve release of cytokines. The effects of different cytokines on metabolic enzymes and/or transporters have been documented earlier. There were no studies on the effect of these inflammatory markers that accompanied transplantation or rejection on pyridoxal kinase activity.

Transplant allogenic group that was not treated with tacrolimus showed the lowest level of pyridoxal kinase mRNA expression and the liver extract showed the lowest activity. Allogenic transplant group livers showed the lowest total protein content, which might explain the lower levels of expression. However the activity is normalized to total protein content and still the activity is significantly lower than all groups. The rejection may lead to down regulating various enzymes in the hepatocytes than the effect of either the surgery itself or the drugs given to prevent rejection like tacrolimus. The tacrolimus treated group as well as autogenic group were also significantly lower in total protein compared to control group, similarly, the expression in allogenic groups were significantly different from control. Tacrolimus as a treatment that prevented rejection did not interfere with pyridoxal kinase expression or the corresponding activity.

In summary, the animal study with different transplant models highlighted the effect of transplantation on total liver protein and more specifically on pyridoxal kinase expression. The allogenic transplantation group who underwent rejection shortly had the lowest mRNA expression of pyridoxal kinase and that suggested lower metabolic rate during or following transplantation. However, the tacrolimus treated allogenic transplant group was not different

from the control or autogenic group which suggested that immunosuppression may restore the pyridoxal kinase functional capacity.

7.0 CONCLUSIONS

7.1 INTRODUCTION

Vitamin B6 deficiency is biochemically expressed as low levels of the bioactive metabolite, pyridoxal 5` phosphate (P5P). Vitamin B6 deficiency has been reported in several solid organ transplant recipients with the highest incidence being observed in small bowel transplant recipients. Nearly 96% of the small bowel transplant recipients have been reported to be deficient in vitamin B6. Clinically, the deficiency has many manifestations and symptoms that can exacerbate as neurological, hematological, as well as metabolic abnormalities. While limited systematic studies have been carried out to evaluate the nutritional status of the transplant recipients, no study to date has evaluated the potential mechanism(s) that might contribute to the observed high incidence of vitamin B6 deficiency in transplant patients. In theory, the nature of the transplantation procedure (trauma of the surgery), the immunogenicity of the transplanted organ, the inflammatory status of the transplant recipient, and the drug treatment protocols used in transplant recipients, might contribute to the observed vitamin deficiency in this population. In this study we evaluated potential mechanisms (increased degradation of P5P due to higher levels of alkaline phosphatases; or decreased formation of P5P due to decreased expression and activity of pyridoxal kinase) that may contribute to such observations

The majority of P5P is formed in the liver by pyridoxal kinase and in the blood it is associated primarily with erythrocytes and plasma albumin. P5P is degraded by alkaline phosphatase in to products that are further oxidized to the terminal urinary byproduct 4-Pyridoxic acid (4-PA). Our studies in small bowel transplant patients documented that higher amount of 4-PA is excreted in these patients compared to normal healthy volunteers. The increased excretion was associated with higher plasma concentrations of alkaline phosphatase. Increased excretion can also be due to lower extent of binding of P5P to albumin, given that the plasma albumin concentrations in the small bowel transplant patients were lower than normal.

Decreased formation of P5P as another possible mechanism was supported by several observations. Potential contribution of some of the medications used in transplant recipients was evaluated by computational approach. Mycophenolic acid and pantoprazole were identified as two potential candidates for further evaluation. Tacrolimus, a common immunosuppressive drugs was identified not likely to be an inhibitor based on computational studies. Further evaluation in cytosol and human hepatocytes indicated that tacrolimus has minimal effect overall on the expression and activity of pyridoxal kinase. Pro inflammatory cytokines, specifically TNF-**α** was observed to down regulate the expression and decrease the activity of pyridoxal kinase in human hepatocytes. The observation of increased pro-inflammatory cytokine levels in the serum of small bowel transplant patients is consistent with the lower levels P5P in this patient population.

Finally, in a preclinical limb transplantation setting, we directly documented that the process of transplantation of an organ, that is not associated with the absorption, distribution or elimination of vitamin B6, it self down regulates the expression and activity of pyridoxal kinase.

In Conclusion, the increased degradation of pyridoxal 5` phosphate due to non-drug related inflammatory processes (increased alkaline phosphatase, decreased serum albumin) in addition to down regulation of pyridoxal kinase due to elevated proinflammatory cytokines may explain the vitamin B6 deficiency observed in transplant patients.

Summary

Hypothesis 1: Plasma pyridoxal 5` phosphate concentrations will be decreased in transplant patients due to increased degradation mediated by elevated plasma alkaline phosphatase concentrations.

- ✓ **Increased urinary excretion of 4PA in small bowel transplant patients supports increased P5P degradation in this patient population.**
- ✓ **Increased serum concentrations of alkaline phosphatase supports its potential contribution to increased degradation of P5P.**
- ✓ **Both serum alkaline phosphatase and 4PA excretion appear to recover towards normal with time post transplantation.**

Hypothesis 2: Free fraction of pyridoxal 5` phosphate will be increased due to lower concentration of albumin in patients or due to displacement by other drugs that are also bound to albumin (Tacrolimus) and this will lead to increased clearance of P5P to 4-PA

- ✓ **Lower albumin level in transplant subjects is consistent with increased free fraction of P5P.**

- ✓ **Albumin concentrations tend to normalize with time post transplantation and this is consistent with reduced 4PA excretion with time post transplantation.**
- ✓ **Tacrolimus did not alter *in vitro* binding of P5P and does not contribute to increased 4PA excretion in urine.**

<u>Hypothesis 3:</u> Plasma pyridoxal 5` phosphate concentration will be decreased in transplant patients due to decreased expression of pyridoxal kinase and/or direct inhibition of pyridoxal kinase involved in its formation by concurrent drugs administered.

- ✓ **Pyridoxal kinase activity was inhibited significantly but minimally by tacrolimus at concentration ≥10ng/ml in cytosol.**
- ✓ **Pyridoxal kinase expression was inhibited by tacrolimus significantly but minimally at 20µg/ml in human hepatocytes**
- ✓ **Chronic administration of tacrolimus at a concentration of 20 ng/ml significantly but minimally decreased the expression of pyridoxal kinase.**
- ✓ **Based on *in silico* testing pantoprazole and MPA appear to be good candidate for inhibition of pyridoxal kinase activity.**

<u>Hypothesis 4</u>: Plasma P5P concentrations will be lower in transplant patients due to decreased expression or inhibition of pyridoxal kinase by increased cytokine levels

The following cytokines were elevated post transplantation (IL-6, IL-8, IL-10, and TNF-α) during the early post-transplant study session. Both IL-6 and TNF- α levels remained

elevated at the later post-transplant session. TNF- α significantly decreased the expression and activity of pyridoxal kinase in primary cultures of human hepatocytes.

<u>Hypothesis 5:</u> The process of transplantation itself will elevate inflammatory markers and will decrease the expression and activity of pyridoxal kinase.

- ✓ **Livers from allogenic Transplant group not treated with tacrolimus showed the lowest level of pyridoxal kinase mRNA expression and lowest activity.**
- ✓ **Livers from allogenic transplant group showed the lowest total protein content among all groups studied.**
- ✓ **Livers from allogenic transplant group treated with tacrolimus were similar to these of autogenic group in expression and activity of pyridoxal kinase**.

Strengths of the proposed study:

1. This study performed a mechanistic analysis of the observed vitamin B6 deficiency.
2. It utilized computational approach, *in vitro* testing and *in vivo* animal and clinical studies to identify potential mechanisms.
3. Identified the potential contribution of inflammatory status on vitamin B6 deficiency.

Limitations of the current work: The project conducted has certain limitations that must be addressed in future studies.

1. The clinical study was performed in a small number of patients. This is due to the nature of the surgical procedure involved and the limited number of patients undergoing small bowel transplantation at any given center. This issue can be overcome in the future by performing multicenter collaborative studies.
2. While the vitamin B6 deficiency has been documented in small bowel transplant patients already, plasma concentrations of P5P were not directly measured in the patients who participated in this study at the time of the study. This can readily be addressed in future studies
3. Direct involvement of alkaline phosphatase in the degradation of P5P has been reported in the literature. In future studies the degradation kinetics of P5P can directly be measured in the patient population studied.
4. Plasma albumin concentration was used as an indirect method of estimating free fraction of P5P in this study. Given that P5P is bound to albumin and to red blood cells, future studies should also directly measure plasma protein binding and red blood cell binding of P5P.
5. The computational approach identified a few of the drugs used in transplant patients as potential inhibitors of pyridoxal kinase. These drugs can be directly tested as inhibitors of pyridoxal kinase using liver cytosol.

6. Additional drugs used by transplant patients can be evaluated for their effect on pyridoxal kinase using the computational approach developed in this work.
7. The effect of tacrolimus and cytokines on the expression and activity of pyridoxal kinase was evaluated *in vitro* in this study. Data on the protein content of pyridoxal kinase will add additional dimension to the observations and facilitate data interpretation.
8. While various cytokines were measured in the clinical study and previous animal studies had documented increased cytokine levels in the animal model used, a direct measurement of the cytokine levels and expression was not performed in the rats used in the current study. This information will provide additional support for the involvement of cytokines in mediating the low levels of P5P.
9. Multicenter studies focusing on nutritional status of transplant patients in general and vitamin B6 levels in particular should also be carried out.

7.2 FUTURE DIRECTIONS

1. There is great interest in further investigations of different micronutrient after organ transplantation. In particular, a systematic evaluation of the vitamin status in organ transplantation deserves more attention.
2. The magnitude of nutritional deficiency is expected to vary with the organ transplanted. Multicenter studies of patients after small bowel transplant should be conducted.

3. Further investigation on the correlation between the results of computational approach and direct activity measurements of pyridoxal kinase should be conducted
4. There could be additive or synergistic effect of tacrolimus and cytokines that has not been analyzed in this study. So the future study should measure the impact of tacrolimus along with cytokines
5. Given that one of the most common side effects of tacrolimus is neurotoxicity, and that vitamin B6 deficiency can also contribute to neurotoxicity, the impact of vitamin B6 deficiency in exacerbating the neurotoxicity of tacrolimus must be investigated.

APPENDIX A

[LIVER HOMOGENATE PREPARATION]

1. Work on ice during all steps.
2. Remove the liver from the animal and put it in a beaker containing homogenization buffer.
3. Remove the buffer to remove excess blood.
4. Blot liver lightly and weigh.
5. Cut the liver into small pieces with scissor.
6. Liver samples (5 gm) were placed in homogenization buffer (50 mM Tris HCl buffer, 1.0 % KCl and 1 mM EDTA, pH 7.4, *and 3**.0 ml per gm liver***) and homogenized using an electrical homogenizer and homogenizing up and down 11 times, keeping vessel on ice.
7. The homogenizer should be moved for 5 sec intervals.
8. Centrifuge 20 min at 10,000 g or 10,000 rpm at 4 ºC depending on rotor speed.
9. Pipette supernatant from previous step into clean tubes.
10. Freeze and store at -80ºC.

Buffers Required:

Buffer:	0.05M Tris HCl	or	7.88 g/l
	1.15% KCl	or	11.5 g/l
	1mM EDTA[$]	or	0.3722 g/l

pH = 7.4 adjusted by NaOH or Glacial Acetic Acid (if Tris Base is used).

1/1000 BHT (5 mg/ml Stock)

1/1000 PMSF (40mg/ml Stock)

$ - EDTA = ethylene diamine tetraacetic acid

* - BHT = Butylated Hydroxytoluene is diluted as 1000 X stock (5mg/ml) in Ethanol. Antioxidant that terminates auto oxidation

** - PMSF = Phenylmethylsulfonyl fluoride is diluted as 1000 X stock (40mg/ml) in Ethanol. Serine protease inhibitor.

Note: Average protein content in human liver is 32 mg/ml.

APPENDIX B

[RT-PCR PROTOCOLS]

Day1: Extraction of mRNA and reverse transcription

1. Remove culture medium from the hepatocyte 6-well plate
2. Add 1 ml Trizol into each well, mix and incubate at room temperature for 5 minutes.
3. Transfer the solution into eppendorf tubes, add 200 µL chloroform to each tube, shake with hands (don't vortex) for 15 seconds, incubate at RT for 2-3 minutes until the two layers separated. Centrifuge at 4^0C, 12000 rpm for 15 minutes
4. Transfer the colorless supernatant to another tube. (Take out the supernatant from top to bottom, slowly pipet up. Usually 450 µL solution can be taken out the 50 µL supernatant is left. Some protein may be between the layers, don't take the protein) Add the same volume of isopropanol (450µL), and then add 10 mg/ml glycogen 1µL (the amount of mRNA from cell sample is very small so we add the beads which bind to RNA and make it visible). Shake with hands. Centrifuge at 4^0C, 12000 rpm for 15 minutes.
5. Pour out the supernatant. Add 75% Alcohol (~800 µL to wash), shake few times. Centrifuge at 4^0C, 12000 rpm for 15 minutes.

6. Remove the supernatant (pour out, be gentle), centrifuge again at 4^0C, 12000 rpm for 5min, pipet to remove residual supernatant. Air-dry 5-10 min until transparent.
7. Add 15 µL water (auto-claved ultra-pure water, dispose the whole tube after use) to each tube. Mix and transfer 11µL sample to another tube (0.3 ml small tubes in line)
8. Add 1µL dNTP and 1µL Oligo dT (12-18t) primer (for binding to poly-A tail) to each sample. (In this step, make master mixture of dNTP and Oligo dT is helpful to decrease variation)
9. Incubate for 5 min at 55-60 ^{0}C in the PCR machine (program: AB) and then put them on ice immediately, wait for at least 2 minutes. (This step is to let AAA bind to TTT. Poly-A might be separated with Oligo dT if temperature drops down slowly.)
10. 5*FS (first strand cDNA) buffer 4 µL, 0.1 M DTT 2.5 µL (proteinase inhibitor, prevent degradation of reverse transcriptase), superscript III 0.5 µL (reverse transcriptase), and then add 7 µL mixture to each tube. (total volume of each sample 11+1+1+7=20µL make master mixture for sample number+1)
11. RT (reverse transcription): 1.5 hr (program: A9). Continue with Real Time PCR or store the samples in -20^{0}C

Day2: Real time PCR

1. Dilute the samples 4-folds with water

2. 20 µL SyBr Green system: (2* master mix 10 µL, water 7 µL, primer 1µL)*(sample number +1), + sample 2µL. Master mix contains enzyme, dNTP, buffer and SyBr green. Be careful: never touch the bottom or upper side of the plate with finger.

3. Cover the film, press each edge with flat plate and let it attach tightly.

4. Centrifuge for twice, for the first time, centrifuge briefly and shake by tapping the edges and centrifuge for 5 min, 2500 rpm.
5. Put the plate in Real-time PCR machine. Set up: the name, volume of the sample and location, save. Choose the “dissociation curve”

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