Relations between Vitamin B6, Tryptophan Metabolites, and Inflammation

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By

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If I have seen further, it is by standing on the shoulders of giants – Sir Isaac Newton

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Abstract

Background: A variety of inflammatory conditions, including cardiovascular disease (CVD) and diabetes, and are associated with both increased kynurenine pathway (KP) activity and low plasma concentrations of pyridoxal 5'phosphate (PLP), the bioactive form of vitamin B6. Inflammatory cytokines induce the first enzyme in the KP; downstream enzymes require PLP as a cofactor to produce immunomodulatory kynurenine metabolites. We hypothesize low plasma PLP observed during inflammation is due to increased PLP utilization by KP enzymes during inflammation. This PLP depletion may lead to health conditions related to PLP-dependent functions such as hemoglobin and neurotransmitter synthesis. Four studies were conducted to examine the relation between PLP, kynurenine metabolites, and inflammation in the context of cardiovascular disease, anemia, diabetes, and cognitive and psychological function.

Methods: Secondary data analyses were conducted on data from Framingham Heart Study Offspring Cohort and Nutrition Aging and Memory in Elders (NAME) cohorts, separately. As an ancillary to NAME, kynurenine metabolites were measured in plasma samples using LC-MS/MS. We similarly measured kynurenine metabolites in plasma, aortic, and lymph node tissues from a porcine model of atherosclerosis to assess the effect of diet and statin use on PLP, kynurenine metabolites, and inflammation.

Results: CVD status and plasma PLP were interactive predictors of anthranilic acid concentration: and 2-fold increase in plasma PLP resulted in 1.06 fold change in plasma anthranilic acid concentration among CVD-free subjects. CVD was associated with higher prevalence of plasma PLP insufficiency (PLP<30nmol/L) but not kynurenine metabolite concentrations. Subjects with PLP insufficiency had greater plasma hydroxyanthranilic acid compared to normal (23%, p=0.02). Anemia prevalence was dependent on both plasma PLP and CRP concentrations: it was highest among those in first PLP tertile category with plasma CRP > 10mg/L. Diabetics had lower plasma PLP and higher xanthurenic acid concentrations than non-diabetics. Plasma PLP and xanthurenic acid concentrations were interactive predictors of insulin resistance as measured by HOMA2-IR. PLP insufficiency and the highest plasma concentrations of xanthurenic acid were associated with the highest insulin resistance. Plasma PLP was associated with the attention function of cognition, but kynurenine metabolites were not associated with any domains of cognition or depression. In a porcine model of atherosclerosis, data were inconclusive. Since we did not observe the expected change in inflammation due to diet or statin use, there were no discernable effects of diet or statin use on kynurenine metabolites in plasma, aortic, or lymph node tissues.

Conclusions: This project demonstrated that anemia is associated with both inflammation and PLP insufficiency. Our findings suggest inflammation associated PLP insufficiency may increase risk of negative health outcomes comorbid with inflammatory disease. Additionally, we found xanthurenic acid and PLP are interactively associated with HOMA2-IR, indicating KP activation may be involved in inflammatory mechanisms underlying diabetes development. We were unable to observe relations between kynurenines and cognition, depression, diet, or statin use. Ultimately, we were unable to observe increased KP activation at sites of inflammation as an underlying cause of inflammation-associated PLP depletion in the context of these conditions. Associations between kynurenine metabolites and inflammatory conditions studied here suggest a more complex relation may underlie these physiologic changes than previously expected.

Chapter 1

Introduction

Background

Vitamin B_6 is a water-soluble vitamin that is relatively ubiquitous in the food system. The best food sources of vitamin B6 are beans, legumes, nuts, seeds, potatoes, non-citrus fruits such as bananas, dairy, meats, beef liver, and fish. It is also present in most multivitamin supplements and in fortified hot and cold breakfast cereals [1]. The RDA for vitamin B_6 in 1.3 mg for women over age 19 years and men between ages 19 and 50 years, and 1.7 mg/day for men 51 years and older [2]. Foods rich in vitamin B6 are commonly consumed in the United States [3]; thus, inadequate intake of vitamin B_6 is rare [4]. Early studies that placed subjects on an artificially vitamin B_6 deplete diet determined that cases of deficiency in vitamin B_6 can manifest as seborrheic dermatitis, microcytic anemia, glossitis, depression and confusion [2, 5].

Vitamin B_6 was discovered as an anti-dermatitis agent after discovering that rats developed skin irritation (dermatitis acrondynia) despite supplementation with Vitamins B1 (Thiamin) and B2 (Riboflavin) [6]. Paul György named this “antidermatitis factor” vitamin B_6, or pyridoxine. The bioactive form of vitamin B_6 in the body is pyridoxal-5’-phosphate (PLP).

The main biochemical functions of PLP are as a Schiff base when bound to the lysine residue of enzymes involved in decarboxylation, transamination and racemization [1]. PLP is used in conjunction with folate, and vitamin B12 in reactions of one-carbon metabolism, which are essential for methylation and remethylation reactions [1]. PLP is also required for transamination of kynurenine into kynurenine metabolites, condensation of glycine with succinyl-CoA in the synthesis of heme, and converting L-DOPA to dopamine. These reactions are essential to

physiological functions such as inflammation, hemoglobin synthesis, and neurotransmitter synthesis [1, 7-9].

PLP and Inflammation

Previous studies indicate that patients with inflammation related diseases such as cardiovascular disease (CVD), rheumatoid arthritis, irritable bowel disease, diabetes, and cancers have low plasma concentration of the bioactive form of vitamin B_6, pyridoxal 5'-phosphate (PLP), despite normal vitamin B_6 intake and metabolism [10-13]. Low plasma PLP is associated with higher concentrations of inflammatory markers, [14-16] and increased disease severity in diseases characterized by periodic flare ups of inflammation such as RA and irritable bowel disease [14, 15, 17]. While rheumatoid arthritis, irritable bowel disease, and cardiovascular disease have different pathophysiologies, inflammation appears to be common to these and other chronic diseases. Higher levels of inflammation were associated with low plasma PLP status in the general population as well, across all levels of vitamin B_6 intake [16, 18].

We know from previous work by Chiang et al. that the fate of PLP during inflammation remains unknown. [19]. This study examined rats induced with rheumatoid arthritis in rats and established that inflammation depletes PLP from plasma and liver, but not the main storage organ, muscle [19]. There have been contradictory findings regarding whether PLP breakdown increases during inflammation. Ulvik et al. suggest an increase in PLP breakdown during inflammation after examining plasma indices of PLP catabolism [20]. However, Chiang et al.

did not observe increased urinary excretion of PLP breakdown products during inflammation [19].

PLP and Tryptophan Degradation via the Kynurenine Pathway

Inflammation is associated with increased tryptophan degradation to immunomodulatory kynurenine metabolites, and reactions that produce most of these metabolites require PLP as a cofactor [21, 22]. The first and rate limiting step of the kynurenine pathway is carried out constitutively in the liver and kidneys by the enzyme tryptophan 2,3, dioxygenase, and by an inducible, extrahepatic form of this enzyme, indoleamine 2,3 dioxygenase (IDO) [23 , 24]. Lipopolysaccharide and CD40 ligands can induce expression of IDO, but the most potent inducer is the pro-inflammatory cytokine interferon-gamma (IFN-γ), secreted by macrophages and dendritic cells [25-28]. In fact, dendritic cells express the complete set of enzymes in the kynurenine pathway, including IDO, highlighting the importance of kynurenine metabolism to immune function for these cells [29].

IDO catalyzes the reaction of tryptophan to formylkynurenine, which is then converted to L-kynurenine by kynurenine formamidase [23]. The ratio of kynurenine to tryptophan in tissues is often used as an indicator of IDO activation and as a marker of increased inflammation [30].

Downstream of IDO, kynureninase and kynurenine aminotransferase are two key enzymes that both require the PLP to further degrade kynurenine into kynurenine metabolites [9, 31, 32] (Figure 1). These enzymes are affected in different ways to PLP insufficiency. One study on healthy adults suggested PLP insufficiency may affect kynureninase to a greater extent than

kynurenine aminotransferase, based on their observations that plasma 3-hydoxykynurenine increased while kynurenic acid decreased in adults on an acute vitamin B-6 deficient diet [33]. This supports earlier work of Midttun et al., who examined the association between hydroxykynurenine and PLP in cardiovascular patients and Ogasawara et al., who examined the reaction velocities of kynurenine aminotransferase and kynureninase under varying PLP concentrations [22, 34]. Ogasawara et al. suggest PLP deficiency reduces activity of kynurenine aminotransferase and kynureninase in extra-mitochondrial cell compartments [34]. This shifts kynurenine metabolism to produce 3-hydroxykynurenine. Since mitochondrial kynurenine aminotransferase is not affected by PLP deficiency and kynureninase appears to be largely inactive in mitochondria, mitochondrial kynurenine aminotransferase then converts 3-hydroxykynurenine to xanthurenic acid [34].

The kynurenine metabolites kynurenic acid, 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid, and xanthurenic acid, all have been demonstrated to have immunomodulatory properties [35]. Of these, kynurenic acid, anthranilic acid, xanthurenic acid and 3-hydroxyanthranilic acid all require PLP for their formation [9, 34]. Because of this, some recent papers have suggested the use of kynurenine concentrations and relative ratios of kynurenine metabolites as indicators of functional vitamin B-6 status [36].

Effect of Kynurenine metabolites on immune system markers

Kynurenine metabolites exert varying immunomodulatory effects [35]. For example, 3-hydroxyanthranilic acid, kynurenine, and 3-hydroxykynurenine may regulate an active immune

system by suppressing activated T-cell proliferation, which is important in the context of allogenic transplantation [37, 38]. 3-Hydroxyanthranilic acid has been demonstrated to induce T-cell apoptosis in cell culture [39, 40]. IDO expression is limited during rheumatoid arthritis, and this could be due to impaired regulatory T-cells functionality due to the epigenetic changes to the promoter for CTLA4, a T-cell regulating compound [41].

Other immunomodulatory effects of kynurenine metabolites include apoptosis or recruitment of other immune cell types. Kynurenine potentially reduces immune cell activation via the induction of apoptosis of neutrophils [42]. Additionally, 3- hydroxyanthranilic acid has been shown to reduce recruitment of leukocytes to endothelial cells via reduction of both monocyte chemoattractant protein-1 secretion and vascular cell adhesion molecule-1expression [39, 40, 43]. Lastly, a study using IDO knockout mice suggests kynurenine pathway activation may play a role in inflammatory cell migration, as these IDO knockout mice had increased inflammatory cell infiltration in liver during diet induced steatohepatitis [44].

CVD

Physiologically, kynurenine metabolites can induce a variety of changes, which may alter the risk of developing CVD. For example, kynurenine has been demonstrated to be a vasodilator, and may play a role in reducing blood pressure, an important component of cardiovascular health [45].

Conversely, some kynurenine metabolites and IDO activation may play a key role in atherosclerosis. Studies in hypercholesterolemic mice found that APOe$^{-/-}$ IDO$^{-/-}$ (IDO^{-}) mice had more aortic lesions, greater plaque area percentage, and more indicators of plaque instability than APOe$^{-/-}$ IDO$^{+/+}$ (IDO^{+}) mice [46]. Plasma analysis of the IDO^{-} mice showed decreased kynurenine concentrations, but no difference in tryptophan when compared to IDO^{+} mice. Cell culture studies on atheroma cells further confirmed that the anthranilic acid mimetic 3,4,-dimethoxycinnamoylanthranilic acid, had decreased inflammatory cytokines (tumor necrosis factor alpha, and IL6 and IL10, among others) when compared to untreated cells. Treatment with L-kynurenine did not produce any effect [46]. These results suggest certain kynurenine metabolites, rather than depletion of tryptophan, are active in the atherosclerotic process [46].

Markers of kynurenine pathway activation are associated with CVD on a population level. CVD patients have been observed to have increased kynurenine to tryptophan ratio and among these patients the ratio has been shown to be a prognostic indicator for mortality post ischemia [47, 48] and a good predictor of coronary events in subjects without CVD [49]. Such findings indicate the kynurenine pathway may play an important role in CVD development

CVD is one of the leading causes of death in the United States [50]. To treat or prevent CVD, the CDC reports that approximately 27% of adults in the United States aged 40 and over, and 40% of adults aged 65 and older, take a cholesterol lowering medication, such as a statin [51]. Statins have been demonstrated to reduce general markers of inflammation, such as C-reactive protein, inhibit release of interferon gamma (IFN-γ), and limit T-cell proliferation through inhibition of the IFN-γ induced expression of major histocompatibility complex-II in human

endothelial cells and macrophages [52-54]. Results from these studies suggest statin-mediated inhibition of these inflammatory markers, namely IFN-γ, also reduce kynurenine pathway activation due to decreased induction of IDO [55].

Diabetes

One kynurenine metabolite in particular, xanthurenic acid, is both sensitive to PLP sufficiency and may uniquely affect glucose regulation through its interactions with insulin. Xanthurenic acid is produced from 3-hydroxykynurenine by kynurenine aminotransferase, a PLP-dependent enzyme. It has long been established that low PLP status can be assessed by increased xanthurenic acid excretion in urine after tryptophan load [56, 57].

There have been many previous studies investigating the possible diabetogenic effects of xanthurenic acid [58-60]. Xanthurenic acid chelates with insulin and the xanthurenic-acid-8-methyl-ether-insulin complex has less glucose controlling activity than native insulin [61] [62]. The increased plasma concentrations of xanthurenic acid observed during PLP deficiency may lead to increased xanthurenic acid -insulin complexes affecting glucose tolerance in inflamed individuals.

Other kynurenine metabolites are also implicated in type 2 diabetes. Studies in rat models of type 2 diabetes have shown decreased plasma tryptophan and kynurenine concentrations in diabetic rats (Otsuka Long-Evans Tokushima Fatty rat and spontaneously diabetic Torii) when

compared to their respective controls [63]. These changes suggest altered kynurenine metabolism may be an indicator for prediabetes. [63].

Kynurenine metabolism is also associated with obesity, a known risk factor for diabetes [64]. This is potentially due to low-grade chronic inflammation in overweight and obese subjects, and has been observed in subjects who have and haven't undergone bariatric surgery [65, 66]. Obesity is also associated with increased kynurenine-3-monooxygenase activity, shifting kynurenine metabolism towards the production of 3-hydoxykynurenine and its products [67]. This is of particular interest given xanthurenic acid is a product of 3-hydoxykynurenine and is secretion of xanthurenic acid is increased in diabetes [34, 68].

Health consequences of low PLP due to inflammation

While low plasma PLP concentration has been correlated with inflammation [12], it is unknown if a lack of available PLP would itself exert adverse health effects. PLP is required as cofactor in over 100 reactions throughout the body [3]. These reactions are integral to important physiological functions, such as synthesis of heme, the oxygen carrying component of hemoglobin; and neurotransmitter synthesis [1]. Functional vitamin B_6 deficiency in inflammation was demonstrated by abnormal results of tryptophan and methionine load tests, which measure xanthurenic acid and homocysteine excretion after being given a large oral dose of tryptophan and methionine [10]. The combination of low plasma PLP observed during inflammation, and the importance of PLP to certain physiological functions have not been yet

been explored with respect to prevalence of potential adverse health outcomes, such as anemia, cognitive impairment, or depression, due to low PLP during inflammation.

Anemia

PLP's involvement in heme synthesis is as a required cofactor for the first and rate-limiting enzyme in the synthesis of heme, δ-aminolevulinate synthase [69]. Inadequate heme leads to anemia: a dearth of fully functional oxygen-carrying red blood cells. The lack of functional red blood cells means oxygen carrying capacity of the blood is limited. In addition to hemoglobin, other hemoproteins such as cytochrome, catalase, and endothelial nitric oxide synthase also rely on heme for their function [1].

Cognitive Conditions

On a biochemical level, changes in cognition can be described through changes in neurotransmitter balance within the brain. PLP is involved in neurotransmitter synthesis is as a cofactor for the enzyme aromatic L-amino acid decarboxylase [8]. This enzyme requires PLP in the decarboxylation reactions to produce neurotransmitters such as dopamine, serotonin, tyramine, and histamine [70]. Additionally, aromatic L-amino acid decarboxylase is also involved in the synthesis of neuromodulators tyramine, tryptamine, and phenethylamine.

Studies have found that low PLP levels are associated with increased prevalence of depression [71, 72]. Despite these findings, there have been no studies to determine if the associations

observed between PLP and depression may depend, at least in part, on utilization of PLP for kynurenine metabolism during inflammation.

Given these possible negative health consequences of low PLP status, it is important to determine if there are ways to prevent inflammation-related PLP deficiency from occurring. Vitamin B_6 deficiency is the leading vitamin deficiency in the United States, according to the Center for Disease Control and Prevention's Second Nutrition Report [73]. Since plasma PLP insufficiency has been observed in the context of inflammatory diseases, rather than inadequate dietary intake, further investigation into the interplay between PLP and inflammation is merited.

Summary

The associations between PLP and inflammatory conditions have been well established. While new associations between kynurenine metabolites and inflammatory conditions are constantly being established, the current body of evidence linking PLP to kynurenine metabolism is still nascent. Here we have summarized studies showing the reduced plasma concentration of PLP during inflammation within human populations, and summarized some of the functions of PLP. This review also summarized the current literature surrounding the role of kynurenines in the etiology of two specific inflammatory diseases: cardiovascular disease and diabetes.

Hypothesis

Patients with inflammatory diseases, such as cardiovascular disease (CVD), have low plasma concentration of pyridoxal-5'-phosphate (PLP), the bioactive form of vitamin B_6, despite normal intake and metabolism. Low plasma PLP may affect PLP-dependent reactions, such as the biosynthesis of heme and neurotransmitters. During inflammation, activity of extra hepatic kynurenine pathway of tryptophan degradation increases and products of PLP-dependent reactions in the pathway are thought to modulate immune response. We ***hypothesize*** that low plasma PLP observed during inflammation is due to mobilization of PLP to sites of inflammation, where PLP is involved in the regulation of the immune response through the kynurenine pathway of tryptophan degradation and that inflammation reduction through statin use could normalize PLP levels. Additionally we hypothesize, reduced PLP in circulation during inflammation is a risk factor for additional adverse health effects often comorbid with inflammatory conditions. We tested this hypothesis by pursuing the following specific aims.

Specific Aims

1. Determine the relationship between CVD, kynurenine products and plasma PLP in a free-living U.S. population. We hypothesize subjects with CVD will have decreased PLP concentration and the association between CVD and PLP will depend, in part, on kynurenine metabolite concentration. To address this hypothesis, we conducted a secondary data analysis on plasma PLP concentration, kynurenines concentrations, and CVD status in the Framingham Heart Study, Offspring Cohort, fifth examination.

2. Determine the relation between inflammation, kynurenine products, plasma PLP, and PLP-related health outcomes in Boston-area elders. We hypothesize subjects with inflammation will have lower plasma PLP concentrations, and that these lower PLP concentrations will be associated with increased prevalence of cognitive impairment, depression, and anemia to test this hypothesis, we used data on plasma PLP and hemoglobin concentration, psychological screening, cognitive testing and analyzed plasma samples to measure kynurenines concentrations in the Nutrition, Aging, and Memory in Elders Cohort.

3. Determine the effect of diet-induced atherosclerosis on kynurenine products, and PLP status in plasma and vascular tissue in a porcine model. We hypothesize an atherosclerotic diet will lead to higher inflammation markers and kynurenine concentrations in tissues and plasma when compared to pigs on a heart healthy diet, or treated with cholesterol-lowering statin, and PLP concentrations to be lower in plasma and higher in inflamed tissues in atherosclerotic pigs. We will examine PLP concentrations in plasma and aortic tissue, to determine PLP distribution across tissues in relation to diet and statin. To test this hypothesis, we measured the concentration of PLP, kynurenines, and markers of inflammation in plasma and aortic tissue samples from a porcine model of diet-induced atherosclerosis.

Significance

CVD and a variety of inflammatory diseases are associated with PLP deficiency and increased tryptophan degradation via the kynurenine pathway. No study thus far has investigated if PLP and the kynurenine pathway are active at the site of inflammation. *The research conducted here has significant novelty as its outcome will advance our understanding of the mechanisms of PLP reactions and kynurenine pathway activation both within free-living, elderly populations, and at sites of inflammation* as determined with a primary atherosclerosis prevention model of diet and statin use in pigs. Additionally, given that inflammation is associated with anemia and depression and PLP is involved in heme and neurotransmitter biosynthesis, this study allowed us to determine whether low plasma PLP observed during inflammation could have additional health consequences [69, 72-76]. Elucidating the role of PLP and tryptophan degradation in inflammation can inform future strategies to reduce the prevalence of inflammation-associated anemia and depression.

To investigate the role of kynurenine pathway activation and PLP at the site of inflammation, this project used samples from Ossabaw pigs. Ossabaw pigs are similar to humans with regard to their cardiovascular physiology, omnivorous diet, development of atherosclerosis due to diet, and response to statins [77]. This project is innovative because it investigated a biological mechanism from multiple approaches, i.e. at both the population level using the circulating markers relevant to disease prevalence and at the atherosclerotic lesion for local inflammation status in animal model that is ideally suited to mimic the pathogenesis of atherosclerosis in humans. This and future studies discerning the role of PLP and kynurenine pathway activation at sites of inflammation.

Figure

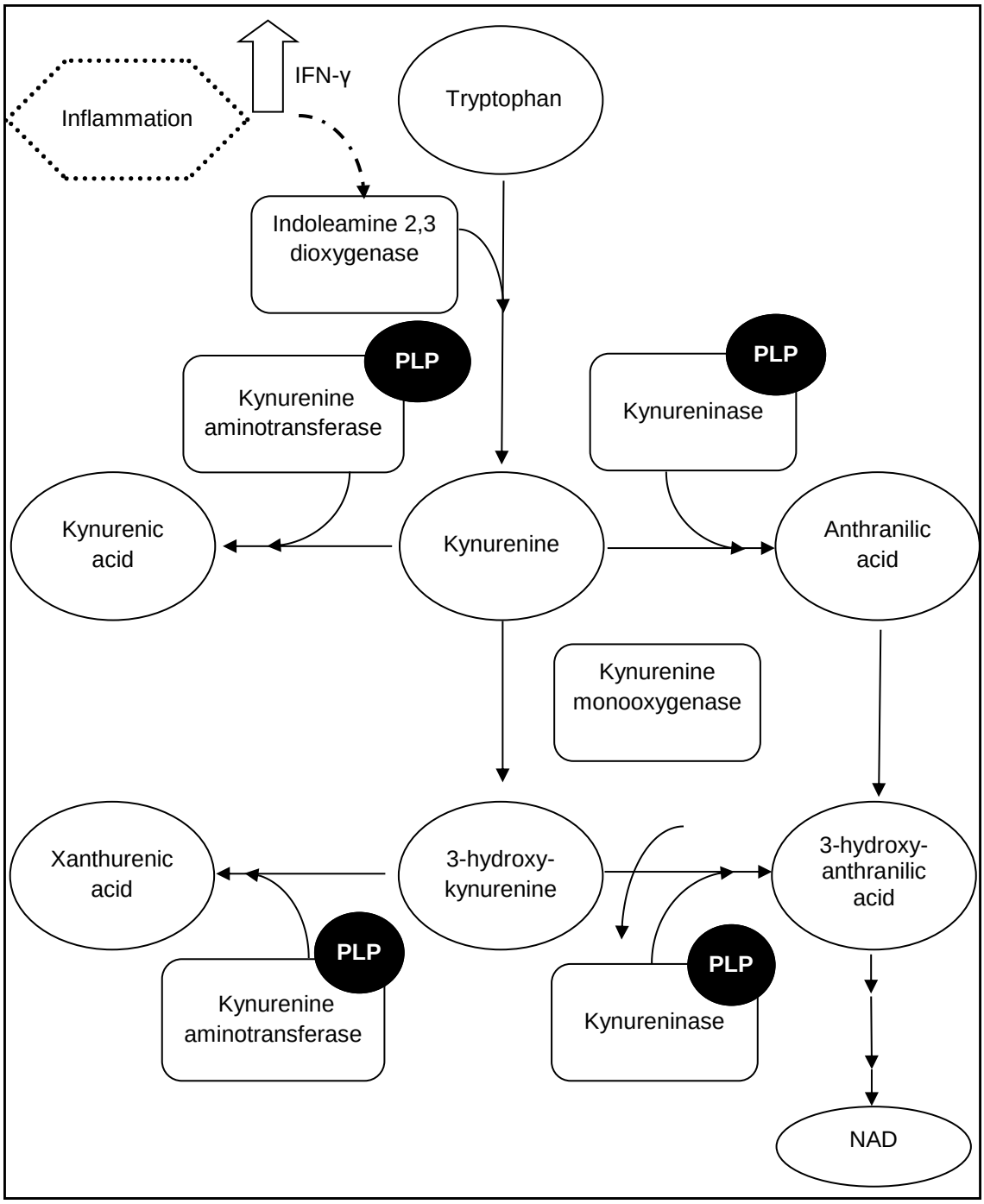

Figure 1: Illustration of The kynurenine Pathway. During inflammation, macrophages and dendritic cells secrete interferon gamma (IFN-γ) which in turn increases expression of indoleamine 2,3 dioxygenase (IDO). Enzymes downstream of IDO require PLP to produce kynurenine metabolites.

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Chapter 2

Plasma Pyridoxal 5'-phosphate (PLP), Inflammation and Tryptophan Degradation Products in Relation to CVD

Abstract

Cardiovascular disease (CVD) is the leading cause of death in the United States. CVD and other diseases characterized by inflammation have been correlated with low plasma concentrations of the bioactive Vitamin B6 vitamer, pyridoxal 5'-phosphate (PLP), independent of Vitamin B6 intake or aberrant B-vitamin metabolism. Increased tryptophan degradation via the kynurenine pathway is also correlated with CVD, and the production of immunomodulatory kynurenine metabolites requires PLP. We hypothesized increased kynurenine metabolite production in subjects with CVD would be correlated with decreased plasma PLP, as a result of PLP utilization in the kynurenine pathway. We conducted a secondary data analysis on data from the Framingham Heart Study to examine the relation between PLP, kynurenine metabolites, and inflammation at Exam 5. Subjects with CVD had higher prevalence of PLP insufficiency (30 nmol/mL) than subjects without CVD (21.2% (95%CI: 8.8 – 12.2) vs 10.5% (95% CI: 15.1 – 27.4), respectively, p-value = 0.002). Subjects with insufficient plasma PLP (<30 nmol/L) had 23% higher plasma HAA concentrations than subjects with normal plasma PLP (≥ 30 nmol/L) (p=0.02). The association between plasma AA and CVD depended on PLP sufficiency, wherein plasma AA was greater in subjects with both CVD and PLP insufficiency than subjects without CVD or with normal plasma PLP. These results suggest increased HAA and AA concentrations are correlated with PLP insufficiency, and that kynurenine metabolite production is dependent on both disease status and PLP availability.

Introduction

Inflammatory diseases, including cardiovascular disease (CVD), rheumatoid arthritis (RA), and diabetes, are associated with low plasma concentrations of pyridoxal-5'-phosphate (PLP),

the coenzyme form of vitamin B_6, independent of inadequate vitamin B_6 intake or aberrant B-vitamin metabolism [1-7]. Numerous studies have determined inverse relationships between plasma PLP levels and risk of coronary heart disease, coronary artery disease, and atherosclerosis, which fall under the class of CVD [2, 4, 8, 9]. Additionally, higher levels of inflammatory markers, including C-reactive protein, interleukin-6, tumor necrosis factor-α, and increased disease severity have been associated with low PLP, indicating plasma PLP is modulated concurrently with inflammatory states, and not a static effect of disease status [10-13]. This could be especially relevant in conditions of chronic low-level inflammation associated with aging.

During inflammation, inflammatory cytokines such as interferon gamma (IFN- γ) increase tryptophan degradation through the kynurenine pathway by inducing extrahepatic indoleamine 2, 3 dioxygenase (IDO) [14, 15]. IDO is the inducible form of the constitutively expressed hepatic protein tryptophan 2,3 dioxygenase. IDO is found in immune cells such as macrophages, dendritic cells, and monocytes [14, 16]. An important aspect of IDO-dependent tryptophan degradation is that PLP functions as a cofactor for two enzymes in the kynurenine pathway. These enzymes produce immunomodulatory kynurenine metabolites such as kynurenic acid (KA), xanthurenic acid (XA), anthranilic acid (AA) and 3-hydroxyanthranilic acid (HAA) [17].

Increased plasma kynurenine to tryptophan ratio (KTR), an indicator of IDO activity, is a risk factor for major coronary events in patients with stable coronary artery disease, a type of CVD also characterized by inflammation [18]. Inverse associations between the kynurenine metabolite 3-hydroxykynurenine and plasma PLP levels among patients suspected to have coronary heart disease with high levels of one or more inflammatory markers [19]. Further evidence of increased kynurenine pathway activation in CVD has been supported by cell culture studies

showing HAA reduces recruitment of leukocytes to endothelial cells via reduction of both monocyte chemoattractant protein-1 secretion and vascular cell adhesion molecule-1expression [20, 21, 22].

The present study was conducted to examine the relation between low PLP and cardiovascular disease in association with kynurenine pathway activation. We propose that the low PLP during periods of inflammation reflects mobilization of this coenzyme to sites where IDO-dependent tryptophan degradation is taking place. We investigated this hypothesis using data originally collected for metabolite profiling [23] of Framingham Heart Study Offspring Cohort participants who attended the 5th study examination to determine if metabolite profiles could predict the development of diabetes [23].

Methods

Study Population:

The Framingham Heart Study Offspring Cohort is described in detail in previous literature [24]. These subjects were offspring of participants in the original Framingham Heart Study cohort, and were recruited starting in 1971. The data used for the present study were collected on 3,799 participants, aged 26 and 84 years old, who attended the 5th study examination, which occurred between 1991 and 1995.

Metabolite profiling was conducted by Wang et al. as part of a metabolite profiling study to examine associations with type 2 diabetes [23]. Of 3,799 attendees to the fifth examination, metabolites were measured on 2,526 participants. Data on kynurenine metabolites was available for 2,076 subjects. After excluding subjects with missing or invalid PLP or covariate data, there

were 1,315 subjects remaining with complete covariate data and plasma KA and AA measurements, and 765 subjects with complete covariate data and plasma HAA measurements. 765 subjects have complete covariate data and all three kynurenine metabolites measured.

Exposures

The main exposures for this project are pyridoxal 5'-phosphate (PLP), the active circulating form of vitamin B6, and prevalent CVD. PLP was measured in the Framingham Offspring cohort at exam five using the tyrosine decarboxylase apoenzyme method [25]. Plasma PLP deficiency was defined as plasma PLP concentrations less than 20 nmol/L, which is the definition used by the Food and Nutrition Board. PLP insufficiency was defined as < 30 nmol/L, a value suggested by previous researchers that has been associated with some adverse health conditions [26-28]. CVD events were ascertained through continuous surveillance. Hospitalization records and physician office visit records are obtained and reviewed by a committee of three experienced investigators. Criteria for the diagnoses of cardiovascular events have been described elsewhere [29]. Prevalent CVD includes coronary heart disease (recognized or unrecognized myocardial infarction, angina pectoris, and coronary insufficiency), cerebrovascular disease (stroke and transient ischemic attack), congestive heart failure, and peripheral vascular disease (intermittent claudication).

Outcomes

Data for our primary exposures, plasma levels of kynurenic acid (KA), anthranilic acid (AA) and 3-hydroxyanthralinic acid (3-OH-AA), were generated as part of a previous metabolite profiling using a liquid chromatography-tandem mass spectrometry (LC-MS). Methods are described in detail by Wang et al [23]. Briefly, plasma samples were analyzed using an high

performance liquid chromatography system (Agilent Technologies 1200 series) attached to a triple quadrupole mass spectrometer (4000 Agilent Biosystems/Sciex) using Atlantis HILIC Silica columns as a stationary phase and 2 mobile phases: 10 mM ammonium formate with 0.1% formic acid (v/v) and acetonitrile with 0.1% formic acid (v/v) [23]. Data for plasma concentrations of AA, HK, HAA are not absolute concentrations – they are normalized to internal standard and pooled plasma [30].

Covariates

A number of covariates were considered as possible confounders for the association between PLP, CVD and KA, AA, or HAA. Self-reported characteristics, such as sex, age, cigarette smoking, and non-steroidal anti-inflammatory drug (NSAID) use, which were collected from general health questionnaire. Subject's weight, height, and waist circumference were measured during clinical examination; body mass index (BMI, kg/m2) calculated from weight and height. Vitamin B6, protein, and energy intakes were collected using semi-quantitative food frequency questionnaire [31]. Concentrations of plasma folate measured using microbiological assay using L. *casei* [32] and plasma vitamin B-12 measured using commercially available BioRad Trilevel Immunoassay kit. Plasma concentrations of homocysteine was measured using HPLC [33], total cholesterol was measured using enzymatic methods [34], and creatinine was measured colorimetrically [35].

Statistical Analysis:

Baseline characteristics are displayed as mean ± standard deviation. Prevalence of plasma PLP insufficiency was calculated as the mean for the PLP insufficiency variable (coded 0=normal, 1=insufficient). Analysis of covariance was used to compare the prevalence of

insufficient PLP amongst those with and without prevalent CVD, adjusting for covariates age, gender, BMI, plasma folate concentration, plasma vitamin B12 concentration, plasma homocysteine concentration, and plasma creatinine concentration.

The relationship between PLP insufficiency and the three kynurenine plasma metabolite concentrations (KA, AA, and HAA) were examined using multiple linear regression adjusting for age, gender, BMI, plasma folate concentration, plasma vitamin B12 concentration, plasma homocysteine concentration, and plasma creatinine concentration. Relative differences in kynurenine concentrations between PLP insufficient and Normal PLP groups means were calculated as (group mean for PLP insufficient) – (group mean for Normal PLP) / (group mean for PLP insufficient). Similar analyses were done to examine the relationship between prevalent CVD and the three kynurenine metabolite outcomes, adjusted for age, gender, BMI, statin use, NSAID use, smoking status, plasma tryptophan. A third set of analyses examined the interaction between PLP insufficiency and prevalent CVD on levels of the three kynurenine metabolites by the addition a cross-product term for PLP insufficiency and CVD to models including both PLP insufficiency and prevalent CVD. In cases where the interaction term was significant ($p<0.05$), the analyses were stratified by prevalent CVD status (yes/no) to determine if the association between PLP and kynurenine metabolites differs among those with and without CVD. These models were also adjusted for age, gender, BMI, statin use, NSAID use, tryptophan, plasma folate concentration, plasma vitamin B12 concentration plasma homocysteine concentration and plasma creatinine concentration. Logarithmic transformations of the dependent variables (KA, AA, and HAA) were applied for formal analysis to meet the assumptions of linear regression and analysis of variance. When dependent variables were log transformed for formal analysis, geometric means of these variables are presented in the scale of the original data. P-values to test

difference in means between groups are calculated from analysis of covariance (ANCOVA), using Turkey's Honestly Significant Difference to adjust for multiple comparisons.

Results

General characteristics of this population are found in Table 1. On average, the subjects in this study were overweight, with an average BMI of 27.6. The geometric mean of plasma folate concentration for this cohort was 13.2 nmol/L, which is the low end of the normal range for plasma folate. This is expected, given that exam 5 occurred between before mandatory folate fortification in the United States. Average vitamin B6 intake was 5.1 mg/day, which exceeds Estimated Average Requirement for all age groups and genders (males and females aged 18-50: 1.1 mg/day, males over 50 1.4 mg/day, females over 50 1.3mg/day). Prevalence of subjects who did not meet age and gender specific Estimated Average Requirement for vitamin B6 was 10.70%. Geometric mean for plasma PLP concentration was in the normal range, 59.9 nmol/L, and the prevalence of plasma PLP deficiency (>20nmol/L) was low in this population, 2.1%.

The prevalence of subjects with insufficient plasma PLP concentration (less than 30 nmol/L) amongst subjects with CVD was more than double that of subjects without CVD (21.2% (95%CI: 8.8 – 12.2) vs 10.5% (95% CI: 15.1 – 27.4), respectively, p-value = 0.002) (Figure 1).

Examination of the multivariable adjusted association between insufficient plasma PLP and the three kynurenine metabolites, demonstrated that HAA was 23% higher in subjects with insufficient PLP vs normal PLP (0.26 (95%CI: 0.21-0.33 vs 0.20 (95%CI: 0.19-0.22) respectively, p=0.02) but KA and AA did not differ between those with normal and insufficient plasma PLP (Table 2), but we did not observe difference for any of the plasma kynurenine metabolites between subjects with and without CVD (Table 3).

In the analyses examining the possible interactions between PLP insufficiency and prevalent CVD, we observed that that the relation between insufficient PLP and plasma AA concentration depended on the presence or absence of prevalent CVD, as indicated by a statistically significant (p=0.01) regression coefficient for the cross-product term between insufficient PLP and prevalent CVD. Among subjects with insufficient PLP, subjects with CVD had 51.7% higher plasma AA concentrations than subjects without CVD (2.61 (95%CI: 2.33-2.93) vs 3.96 (95%CI: 2.95-5.33), respectively, p=0.01) (Table 4).

Discussion

The data presented here support previous findings that inflammatory conditions are associated with low plasma PLP concentrations. We also found an inverse association between PLP and HAA – average HAA concentrations were higher in subjects with low plasma PLP status. The association between AA and CVD depended on PLP status: among subjects with insufficient PLP subjects with CVD had much higher plasma AA concentrations, whereas there was no association between found in subjects with normal plasma PLP. This could be due to differential activation of the kynurenine pathway depending on inflammatory state and PLP availability.

There are two enzymes in the kynurenine pathway that require PLP as a cofactor: kynureninase, which produces AA and HAA; and kynurenine aminotransferase, which produces KA. KA was not found to be associated with PLP status nor CVD status, whereas the association between AA and PLP depended on inflammatory state – in this case the presence or absence of CVD. Given that lower PLP was observed in subjects with CVD, the observed associations between PLP status and AA as well as its metabolite HAA support the possibility that kynureninase may be more affected by depletion of PLP during inflammation than kynurenine

aminotransferase, which produces KA. This was suggested by previous studies [28, 36]. These findings reveal a complexity between PLP status and the production of kynurenine metabolites, that may be both useful in further understanding both interrelations between the two, as well as their separate influences on inflammation and health.

In the present study, the prevalence of PLP insufficiency (<30 nmol/L) was twice as high in subjects with CVD as in those without, which is in agreement with many previous studies [2, 9]. The public health ramifications of the increased prevalence of PLP insufficiency have not yet been explored, and given the that CVD is the leading cause of death in the United States [37], this warrants further investigation. Due to the cross-sectional nature of this study, we are unable to come to any certain conclusions regarding causality; however the findings of increased HAA in PLP insufficient subjects, as well as the relation of AA with CVD status depending on plasma PLP status supports the hypothesis that increased kynurenine pathway activation in CVD may underlie lower plasma PLP concentrations. These result warrant further investigation into this association, preferably with a greater number of kynurenine metabolites. We conclude that increased production HAA is observed in subjects with low plasma PLP, and the relation between PLP and AA depends on both availability of PLP and disease status.

Figures and Tables

Table 1: General Characteristics of the Framingham Offspring Cohort at Exam 5

Table 1	**N=1,315**	
Characteristic	**Mean**	**(SD)**
Age (yrs)	55.43	(9.59)
Female n(%)	652	(49.58%)
BMI (kg/m^2)	27.60	(4.86)
Pyridoxal Phosphate (nmol/L)*	59.87	(1.83)
Vitamin B6 intake (mg)	5.08	(15.25)
Vitamin B6 intake below EAR† (n,%)	82	(10.70%)
Homocysteine (µmol/L)	10.09	(3.67)
Plasma folate (nmol/L)*	13.17	(4.58)
Vitamin B-12 (pg/mL)*	400.57	(1.67)
Creatinine[1]	1.06	(0.21)
Waist Circumference	36.90	(5.47)
Total Cholesterol	206.77	(37.60)
Total Calories (kcal/day)	1880.44	(619.69)
Protein intake (gm/day)	77.70	(28.22)
Current smoker (n,%)	246	(18.71%)
NSAID use (n,%)	154	(11.71%)
Statin use	48	(3.65%)
Deficient B6 intake	204	(15.51%)
Smoker	246	(18.71%)
PLP deficiency**	28	(2.13%)
Prevalent CVD	99	(7.53%)

***** geometric mean presented; ** (<20 nmol/L),
† Estimated Average Requirement

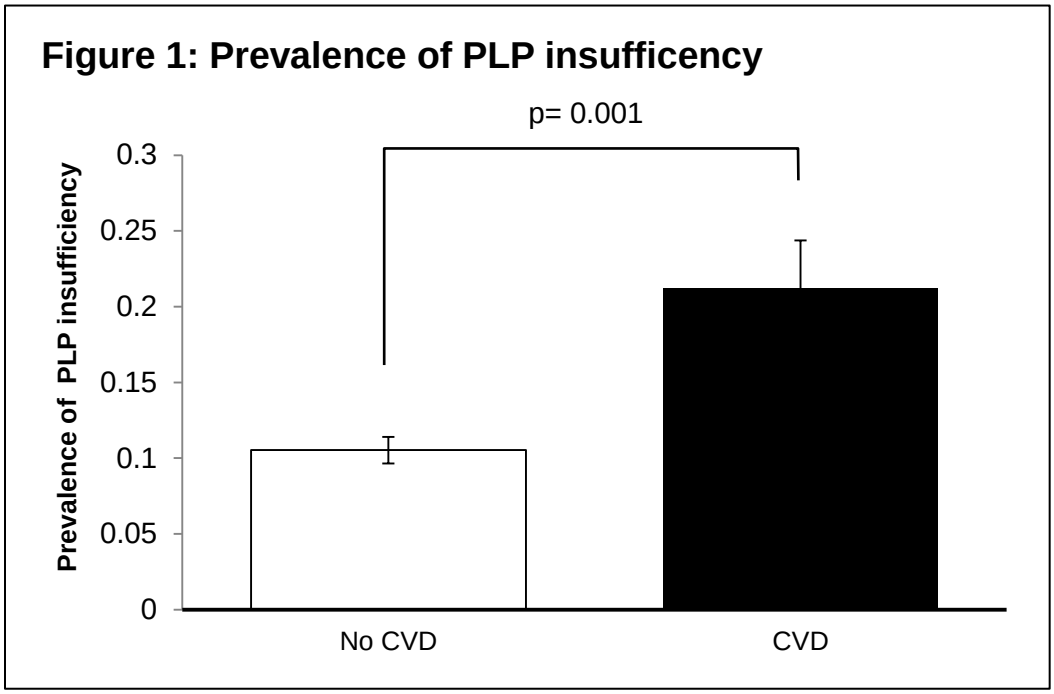

Figure 1: Prevalence of insufficient plasma PLP (>30 nmol/L) amongst patients with and without CVD. n=1,315. Calculated from adjusted means of dummy PLP insufficiency variable parameterized as 1 for CVD and 0 for no CVD. Adjusted for age, gender, BMI, plasma folate concentration, plasma vitamin B12 concentration plasma homocysteine concentration and plasma creatinine concentration.

Table 2: Differences in select kynurenine metabolites across PLP levels

	Unstratified	Insufficient PLP (<30nmol/L) Mean (95% CI)	Normal PLP (≥30 nmol/L) Mean (95% CI)	p-value	Relative Difference**
AA*	3.05 (2.87-3.23)	2.80 (2.53-3.10)	2.94 (2.84-3.04)	0.37	-5.0 %
KA*	1.20 (1.16-1.25)	1.01 (0.94-1.10)	1.08 (1.06-1.12)	0.11	-6.9 %
HAA*	0.34 (0.32-0.37)	0.26 (0.21-0.33)	0.20 (0.19-0.22)	0.02	23.1 %

* AA: n=1315; KA: n=1315; HAA n=766

** Inadequate PLP compared to Normal PLP

Geometric means of plasma kynurenic acid (KA) anthranilic acid (AA) and hydroxyanthranilic acid (HAA), presented in original scale (standardized units). Adjusted for covariates: age, gender, BMI, smoking status, plasma folate concentration, plasma vitamin B12 concentration plasma homocysteine concentration and plasma creatinine concentration. P-value from ANCOVA after adjusting for covariates.

Table 3: Differences in kynurenine metabolites in those with and without CVD

	Unstratified	CVD Mean (95%CI)	No CVD Mean (95%CI)	p-value	Relative Difference**
AA*	3.05 (2.87-3.23)	3.24 (2.86-3.66)	2.90 (2.80-3.00)	0.09	11.7 %
KA*	1.20 (1.15-1.25)	1.06 (0.96-1.18)	1.08 (1.05-1.11)	0.79	-1.9 %
HAA*	0.34 (0.32-0.37)	0.22 (0.16-0.29)	0.21 (0.19-0.22)	0.71	4.5 %

* AA: n=1315; KA: n=1315; HAA n=766

** CVD compared to No CVD

Geometric means of plasma kynurenic acid (KA) anthranilic acid (AA) and hydroxyanthranilic acid (HAA), presented in original scale (standardized units). Adjusted for covariates: age, gender, BMI, statin use, NSAID use, smoking status, plasma tryptophan. P-value from ANCOVA comparing No CVD to CVD after adjusting for covariates.

Table 4: Differences in AA concentrations by CVD and PLP status

	Unstratified	CVD Mean (95%CI)	No CVD Mean (95%CI)	p-value	Relative Difference**
Insufficient PLP	3.07 (2.88-3.27)	3.96 (2.95-5.33)	2.61 (2.33-2.93)	0.01	51.7 %
Normal PLP	2.88 (2.33-3.42)	3.08 (2.69-3.54)	2.94 (2.83-3.04)	0.51	4.8 %

*Cross-product term (CVD prevalence * PLP) p= 0.00766 n=1315;

** CVD vs No CVD

Geometric means of plasma anthranilic acid (AA), presented in original scale (standardized units). Adjusted for covariates: age, gender, BMI, smoking status, statin use, NSAID use, tryptophan, plasma folate concentration, plasma vitamin B12 concentration plasma homocysteine concentration and plasma creatinine concentration.

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Chapter 3

Plasma Pyridoxal 5'-phosphate (PLP), Inflammation, and Tryptophan Degradation Products in Relation to Type 2 Diabetes

Abstract

Diseases characterized by inflammation such as type 2 diabetes are correlated with low plasma concentrations of the bioactive form of vitamin B6, pyridoxal 5'-phosphate, independent of vitamin B6 intake or aberrant B-vitamin metabolism. Inflammation is also associated with increased tryptophan degradation via the kynurenine pathway. Kynurenine metabolites have immunomodulatory properties, and increased urinary excretion of the metabolite xanthurenic acid (XA), has been observed in diabetics when compared to healthy controls. Kynurenine metabolites, including XA, require PLP for their production, yet to date it is uncertain if increased kynurenine pathway activation in diabetics may explain, at least in part, the lower plasma PLP concentrations observed. We investigated the association between plasma PLP, kynurenine metabolites, insulin resistance as indicated by HOMA2-IR, and diabetes status in an ancillary study of the Nutrition, Aging, and Memory in Elders study. Kynurenine metabolites were measured in plasma samples using LCMS/MS. In our cohort we found diabetics had both lower plasma PLP concentrations and 7% higher AA, 26% higher KA, and 19% higher plasma XA concentrations than non-diabetics ($p=0.02$, <0.01, and <0.01, respectively). While plasma PLP was not correlated with plasma XA, the association between XA and insulin resistance depended on PLP sufficiency. HOMA2-IR scores were highest amongst subjects in the highest tertile of plasma XA and PLP insufficiency. These results suggest that increased kynurenine metabolites such as AA, KA and XA are associated with diabetes, and mechanisms that relate XA and PLP to diabetes may be independent.

Introduction

Diabetes has been shown to be associated with low plasma pyridoxal 5'-phosphate (PLP) concentrations [1-4]. Metabolic risk factors for diabetes such as dyslipidemia, obesity, and insulin resistance are also associated with an increase in certain tryptophan degradation products produced via the kynurenine pathway [5, 6]. These kynurenine metabolites have demonstrated immunomodulatory properties [7-9]. Enzymes that convert kynurenine and hydroxykynurenine into immunomodulatory metabolites require PLP as a cofactor. One important kynurenine metabolite is xanthurenic acid (XA).

Increased urinary excretion of XA after a tryptophan load is a hallmark of PLP deficiency [10, 11]. This may seem counterintuitive, given that the enzyme that produces XA, kynurenine aminotransferase, requires PLP as a cofactor (Figure 1). In the kynurenine pathway, kynurenine aminotransferase converts kynurenine into kynurenic acid (KA) and 3-hydroxykynurenine into XA. Kynureninase competes with kynurenine aminotransferase for kynurenine and 3-hydroxykynurenine as substrates. Kynureninase converts kynurenine into anthranilic acid, and 3-hydroxykynurenine to 3-hydroxyanthranilic acid [12-14]. Early studies examining the relative activities of the two enzymes suggest that kynureninase across the different cell compartments is more sensitive to the unavailability of PLP than kynurenine aminotransferase [13]. Previous studies have found increased urinary excretion of XA and higher plasma concentrations of KA and XA have been observed in diabetics [15, 16], supporting the hypothesis kynurenine pathway activation is increased in diabetes. However, these studies did not examine the interrelation between kynurenine pathway metabolites and PLP status [17]. Given KA and XA are products of kynurenine aminotransferase, it is possible that the

increased kynurenine pathway activation is affected by low plasma PLP concentrations, driving an increase in XA and KA metabolites.

In this aim, we sought to examine the interrelation between plasma PLP concentration, XA concentration, diabetes status, and insulin resistance. We hypothesize that during inflammation, increased kynurenine pathway activation to produce metabolites such as XA is related to lower PLP concentrations and results in a higher risk of insulin resistance and diabetes. The objective of the present study is to determine if the relationship between XA and both insulin resistance and diabetes is dependent on PLP status, which would suggest a possible mechanism by which plasma PLP depletion could be related to the development of type 2 diabetes.

Methods

Study Population

Data and plasma samples collected in 2003-2007 from the Nutrition Aging and Memory in Elders study were used for this analysis [18]. Participants aged 60 and over were recruited into NAME through Aging Services Access Points (ASAP), agencies that manage home care services for elders from Boston. To be eligible for ASAP, individuals had to be low-income (<$18,890) residents of Boston, aged 60 and over, and require assistance with critical activities of daily living. Individuals from ASAP were excluded from NAME if they were severely vision or hearing impaired, illiterate, refused or had a caregiver refuse participation, did not speak English, or if they ever had any of the

following conditions: HIV/AIDS, epilepsy, schizophrenia, bipolar disorder, mental retardation, severe cognitive impairment (Mini Mental State Exam score < 9), or brain tumor. Informed consent was obtained from every participant and the NAME study protocol was approved by the Institutional Review Board at Tufts Medical Center.

Trained research staff conducted interviews and took anthropomorphic measurements, over the course of 3 in-home visits. Information collected included age, education, occupation, family size, migration and acculturation, household income, sources of social support, food security, a detailed neuropsychological evaluation, health history questionnaires, and dietary assessment via semi-quantitative food frequency questionnaire [18-23]. One fasting blood sample was collected in K_2EDTA tubes and centrifuged in-home to obtain separated red blood cell and plasma samples. Samples were stored on ice during transport to the Nutrition Evaluation Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts within 2 hours of collection.

Samples were stored at -80°C until analysis of blood metabolites, B vitamins, and hematological measures.

Laboratory analyses

Defining insulin resistance and diabetes: Fasting plasma glucose was measured using kinetic enzyme assay [24, 25]. Fasting plasma insulin was measured using radioimmunoassay (Milipore, St Louis, MO). Insulin resistance was assessed from fasting glucose and insulin levels using the most recent iteration of the homeostatic model assessment method for determining insulin resistance (HOMA2-IR) calculated as

described by Levy et al. [26]. HOMA2-IR improves upon the original HOMA-IR linear equation to approximate insulin resistance by accounting for greater physiological variation and increasing plasma glucose concentrations [26]. Diabetes status was determined by categorizing subjects as having diabetes if they self-reported a previous diagnosis of diabetes, took glucose controlling medications, or had fasting plasma glucose concentrations >126 mg/dL.

Plasma PLP: PLP was measured using a tyrosine decarboxylase apoenzyme assay [27]. We used a previously established cutoff of < 30 nmol/L to define PLP insufficiency [28].

Xanthurenic Acid and other Kynurenine Metabolites: A modified protocol based on the methods described by Midttun et al. was used to measure plasma xanthurenic acid simultaneously with other tryptophan metabolites (kynurenic acid, kynurenine, anthranilic acid, 3-hydoxykynurenine 3-hydroxyanthrnailic acid, anthranilic acid, tryptophan) [29]. Thawed plasma samples were deprotonated using a 1:1 ratio of plasma to 6% (w/v) trichloroacetic acid that was spiked with 100 nmol/mL xanthurenic acid-d4 standard (XA-d4, deuterated xanthurenic acid) (US Biological, Salem, MA). The mixture was immediately vortexed, and shaken at 4°C for 1 hour. Samples were then centrifuged at 13,000 rpm for 30 minutes. The resulting supernatant was filtered through 0.22 μm Durapore® filter plates (Milipore, Cork, IRL) and sample was loaded into glass vial inserts. The method used 5 uL injection volume of sample for LC/MS-MS analysis. An Agilent 1300 series ultra-high pressure liquid chromatography (UHPLC) system was equipped with a degasser, in-line filter (2 mm, 0.2 μm, 70x 0.12mm), Zorbax Eclipse XDB-C8 Rapid Resolution HT reversed-phase C-8 column (2.1 x 50 mm, 1. 8 μm) with

Poroshell 120EC-C8 2.1 mm UHPLC guard column and thermostated column compartment set to 40°C. The UHPLC system was used with an AB Sciex 5500 linear ion trap tandem mass spectrometer with TurboSpray ion source. The mobile phase solutions were A: 650 mM acetic acid, B: 100 mM heptafluorobutyric acid, and C: 90% (v/v) acetonitrile in water. A thermostated autosampler compartment kept samples at 4°C. Mobile phase solutions were delivered through the system at a constant flow rate of 0.4 mL/minute in the following ratios at noted time points: 0 minutes: 98% A 2% B; 2.2 minutes: 78%A 2% B 20% C; 2.3 minutes: 60%A 2% B 38% C; 3.3 minutes: 40% A 2%B 58% C; 3.9 minutes: 2%A 2%B 96% C; 4 minutes: 98% A 2% B; 6 minutes 98%A, 2%B. The total run time was 6.5 minutes. The mass spectrometer was set to positive polarity in multiple reaction monitoring mode, using an ion source temperature of 550°C and voltage of 5500 V. Plasma XA concentration was calculated using the ratio of peak area for XA-d4 to XA. Mass spectrometer parameters for detecting XA-d4: Q1 mass was 210.19 da and Q3 mass was 163.70 da with declustering potential of 45, collision energy of 30 and CXP 12. For XA: Q1 was 206.1da and Q3 was 178.20 da.

Other Laboratory Analyses: Plasma concentrations of folate and vitamin B12 were measured using Immulite Chemiluminescent Assay, and total homocysteine measured using HPLC [30]. Plasma creatinine was measured using a Roche Cobas Mira (F. Hoffmann-La Roche Ltd, Basel Switzerland) and an Olympus AU 400e (Olympus America Inc., Center Valley, PA, USA) [31].

Dietary Assessment

Dietary intakes were assessed using the Harvard semi-quantitative food frequency questionnaire (FFQ) [19-23]. The FFQ consists of a list of foods with a standard serving size and a selection of 9 frequency categories ranging from never or <1 serving/month to ≥ 6 servings/day. The FFQ was interviewer-administered in subjects' homes, which allowed specific details about food items and vitamin supplements to be obtained and confirmed.

Covariates:

A number of variables were considered as potential confounders of the associations examined. These included age, sex, BMI, smoking status (never, former, current), current NSAID use, highest education level, hypertension, plasma folate, plasma total homocysteine, plasma creatinine, and dietary intakes of vitamins B6 and vitamin B12 and energy. Highest education level categorized into 6 levels including elementary school (grades 0-4), middle school (grades 5-8), high school (grades 9-11), high school graduate, some college, bachelor's degree, or graduate school. Hypertension was based on based on self-report of previous hypertension diagnosis, use of anti-hypertensive medications, or average systolic / diastolic blood pressure readings above 140/90 at the initial home visit.

Statistical Methods:

Data were analyzed using SAS v. 9.3 (Cary, NC). All hypotheses tested at the 0.05 level of significance. Plasma PLP and XA concentrations were log transformed where necessary to meet statistical assumptions, in which case formal analysis was performed on the log transformed variables and data is presented as geometric means in the original

scale. Subject characteristics were described using means (95% confidence intervals) for continuous characteristics and numbers (percent) for categorical characteristics. To examine the association between PLP and XA concentrations and diabetes prevalence, average plasma PLP and plasma XA concentrations were calculated for diabetic and non-diabetic subjects using analysis of covariance after adjusting for age and sex.

To examine the relationships between insulin resistance and plasma PLP and XA concentrations, and to test our hypothesized interaction between these two factors in relation to insulin resistance, we characterized subjects PLP insufficient and PLP sufficient as defined above. In order to avoid potential confounding by diabetes medication use, or aberrant insulin resistance as a result of diabetes, we excluded all subjects with diabetes from the insulin resistance analyses. Analysis of covariance was used to compare HOMA2-IR across tertiles of plasma XA concentration stratified by PLP using multiple linear regression, adjusted for age, sex, BMI, smoking status, NSAID use, vitamin B6 intake, total caloric intake, plasma folate, plasma homocysteine, and plasma creatinine. Test for trend was conducted treating the median XA values within the tertile categories as a continuous predictor for HOMA2-IR.

Results

There were 704 total subjects with complete data for the present analysis. The cohort was, on average (mean ± SD), 76 ± 8.3 years old, with BMI 31.6 ± 8.1, and 78.47% female, 12.75% current smokers, and a large proportion of people of white race (61.2%), followed by black (37%), and other races (1.8%). In this cohort, 272 subjects (38.5%) were categorized as diabetic (Table 1).

We first sought to examine the relationship between plasma PLP and XA concentrations. No correlation was found between these factors (partial corr: 0.04, p=0.29) after adjusting for age, sex, BMI, smoking status, tryptophan intake, plasma folate, plasma homocysteine, plasma creatinine.

We next examined the relationship between plasma PLP and XA concentrations and diabetes prevalence. We observed that diabetics on average had 14.2% lower plasma concentrations of PLP (p=0.002) (Figure 2A) and 24.0% higher plasma XA concentration (p<0.0001) (Figure 2B) than non-diabetics. The association between plasma PLP and XA concentrations did not differ between diabetics and non-diabetics (data not shown).

Finally, we examined the relationship between insulin resistance assessed using HOMA2-IR and PLP insufficiency and XA tertile categories to test our hypothesis of an interaction with these two factors. Non-diabetic subjects were divided into two groups, PLP insufficient (n=123) and PLP sufficient (n=307) using the same plasma PLP concentration cutoffs described above. Plasma XA concentration was divided into tertiles categories: <11.19 nmol/L, 11.19 - 18.10 nmol/L and >18.10 nmol/L. Based on a statistically significant interaction term for PLP insufficiency and XA tertile (p=0.04), we stratified the relationship between HOMA2-IR and XA tertile categories by PLP insufficiency status. For the lower 2 tertiles of plasma XA concentration, subjects with PLP sufficiency and insufficiency had similar average HOMA2-IR scores (Table 2). However, in subjects highest tertile category of plasma XA concentration, those with PLP insufficiency had significantly higher HOMA2-IR than subjects who were PLP sufficient.

The interrelation between plasma PLP, plasma XA, and HOMA2IR is graphically depicted in Figure 3. The positive trend in mean HOMA2-IR observed across tertile categories of XA was statistically significant for subjects with PLP insufficiency, (β: 0.044, p=0.001), but there was no trend between HOMA2-IR and XA among subjects with sufficient plasma PLP (β: 0.011, p=0.16).

Discussion

In this cohort, plasma XA and plasma PLP were not associated. This could potentially be due to the cross-sectional nature of our study, which prohibits our ability to observe a longitudinal association between lowered PLP concentrations and XA production. Our results do not support the hypothesis that lower PLP observed in inflammation is due to increased kynurenine pathway activation to produce XA. We were still able to demonstrate an interaction between PLP and XA for insulin resistance. Specifically, there was a positive relation between plasma XA concentrations and insulin resistance in PLP insufficient subjects, but no relation was observed in PLP sufficient subjects. However, given our failure to see any relationship between PLP and XA concentrations, the mechanistic basis for the observed correlations between insulin resistance and both XA and PLP concentrations remains unclear.

Additionally, our results were in agreement with the previous studies reporting low plasma PLP in subjects with inflammatory conditions [1, 32, 33], because in the current study diabetic participants had 14% lower plasma PLP concentration compared to non-diabetic participants. Unfortunately, the mechanism behind this observation needs to be elucidated in further studies.

Recently, Oxenkrug put forth the hypothesis that low PLP status shifts kynurenine pathway metabolism towards XA production [34]. Early work by Ogasawara suggest that kynureninase may be more sensitive to PLP deficiency to kynurenine aminotransferase, leading to increased production of XA and KA in PLP deficiency [13]. Our results do not support this hypothesis, since we observed decreased PLP concentrations and increased XA concentrations in diabetics, yet could not find an association between plasma PLP and plasma XA.

Similar to our own findings, Oxenkrug et al. also found that XA, as well as kynurenine and KA were higher in diabetics than non-diabetics [16]. These results build upon observations first reported by Hattori et al. wherein diabetic subjects had greater urinary excretion of XA/creatinine ratio than normal subjects [15] suggesting that kynurenine pathway activation is increased in diabetes. Further research is necessary to determine the mechanism by which kynurenine metabolites, particularly XA and possibly KA, may be involved in diabetes pathology.

The results of this study are limited by the fact that the cross-sectional nature of the data collection precludes our ability to infer temporality of the relationships between insulin resistance, PLP and XA or draw definitive conclusions of causality. Additionally, while we categorized subjects as diabetic or not-diabetic on the basis of blood glucose concentrations, self-reported previous diagnosis, or self-reported diabetes medication intake; it is prudent to remember that a lack of diabetes diagnosis does not infer a lack of potentially confounding inflammatory processes. Given that this cohort was recruited from recipients of home-care services, all individuals in this cohort are likely afflicted by other inflammatory conditions that have resulted in the necessity for these individuals to

require in-home assistance thru ASAP. As an example, 86.1% of NAME participants reported having some form of arthritis.

The results of this project allowed us to confirm that diabetes status was associated with lowered plasma PLP and higher plasma XA concentrations. Additionally, we determined that subjects dually affected by PLP insufficiency and increased plasma XA concentration also had increased insulin resistance. The results presented here warrant additional research into the combined effects of PLP and XA on type 2 diabetes development.

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Figures and Tables

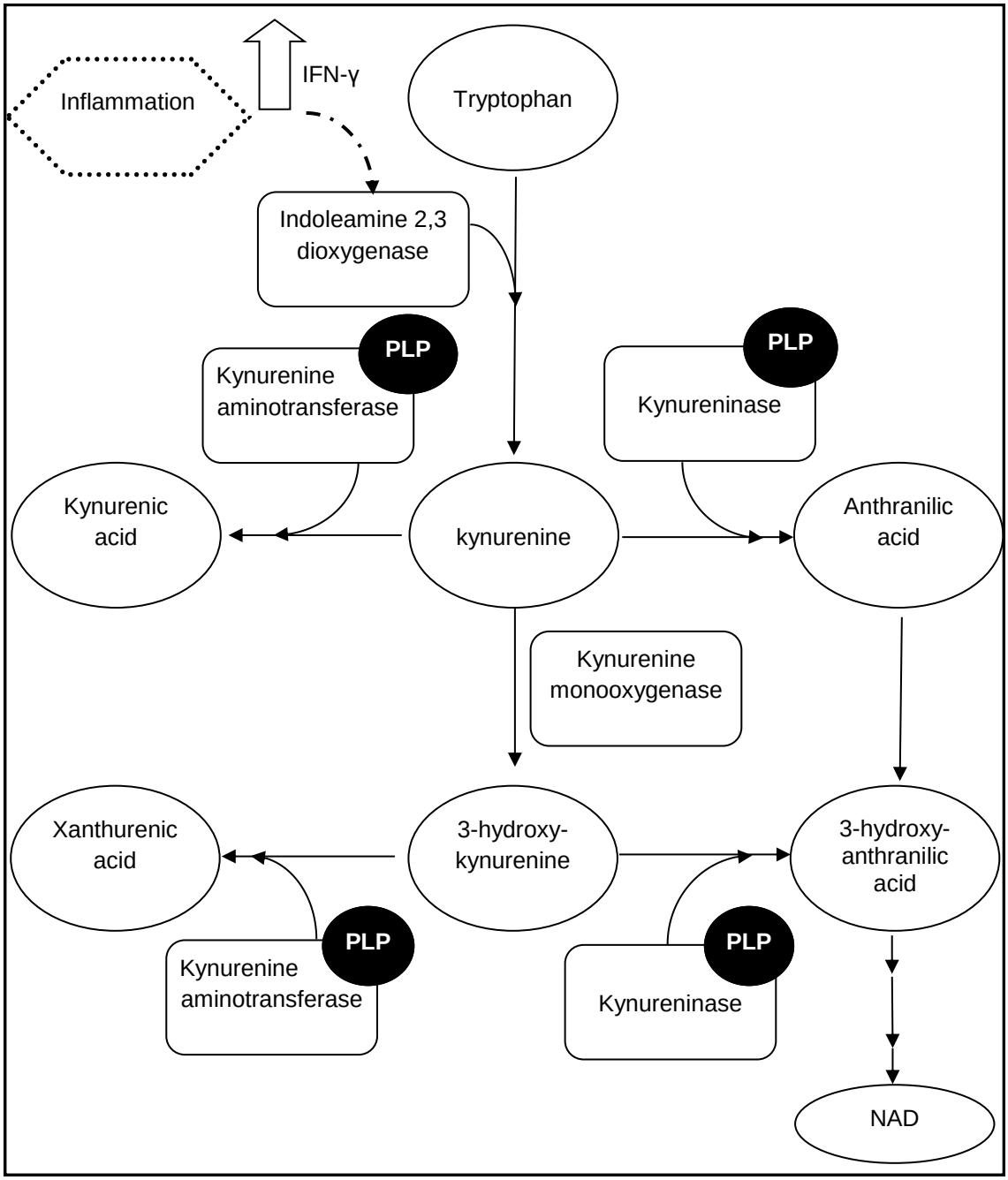

Figure 1: Illustration of The kynurenine Pathway. During inflammation, macrophages and dendritic cells secrete interferon gamma (IFN-γ) which in turn increases expression of indoleamine 2,3 dioxygenase (IDO). Enzymes downstream of IDO require PLP to produce kynurenine metabolites.

Table 1. General characteristics of the Nutrition, Aging, and Memory in Elders cohort

	(n=704)
Characteristic	mean (95% CI)
Age (yrs)	76.25 (75.6-76.9)
BMI (kg/m^2)	31.57 (31.0-32.2)
Pyridoxal Phosphate (nmol/L)	62.44 (57.8-67.1)
Plasma folate (ng/mL)	14.93 (14.3-15.5)
Vitamin B-12 (pg/mL)	610.83 (568.4-653.2)
Homocysteine (μmol/L)*	11.85 (11.5-12.3)
Creatinine	1.09 (1.0-1.2)
Vitamin B-6 intake (mg)	6.40 (5.1-7.7)
Xanthurenic acid (nmol/L)	22.41 (20.5-24.3)
Characteristic	n (%)
Female	554 (78.47)
Current smoker	90 (12.75)
NSAID use	93 (13.17)
Diabetes	272 (38.5)
Low PLP (<30 nmol/L)	212 (30.2)
Race	
Non-Hispanic White	693 (61.19)
Non-Hispanic Black	261 (36.97)
Other race	13 (1.84)

NSAID – Nonsteroidal anti-inflammatory drug;

Table 2 – Association of kynurenine metabolites with Diabetes

	No Diabetes Mean (S.E.)	Diabetes Mean (S.E.)	p-value
Anthranilic Acid (nmol/L)	19.72 (0.39)	21.22 (0.50)	0.02
Kynurenic Acid (nmol/L)	42.90 (1.18)	53.06 (1.50)	<0.01
Hydroxykynurenine (nmol/L)	40.90 (3.23)	39.41 (4.11)	0.77
Xanthurenic Acid (nmol/L)	17.97 (0.72)	21.31 (0.91)	<0.01
Hydroxyanthranilic Acid (nmol/L)	31.08 (1.08)	33.50 (1.38)	0.17

Means in nM/L (standard error) . Adjusted for covariates: age, gender, BMI P-value from ANCOVA after adjusting for covariates.

Table 3 – Association of kynurenine metabolites with insulin resistance

	Change in HOMA2-IR score per nmol/mL increase	**p-value**
Anthranilic Acid	-0.003	0.68
Kynurenic Acid	0.008	<0.01
Xanthurenic Acid	0.010	<0.01

Adjusted for covariates: age, gender, BMI , P-value from ANCOVA after adjusting for covariates.

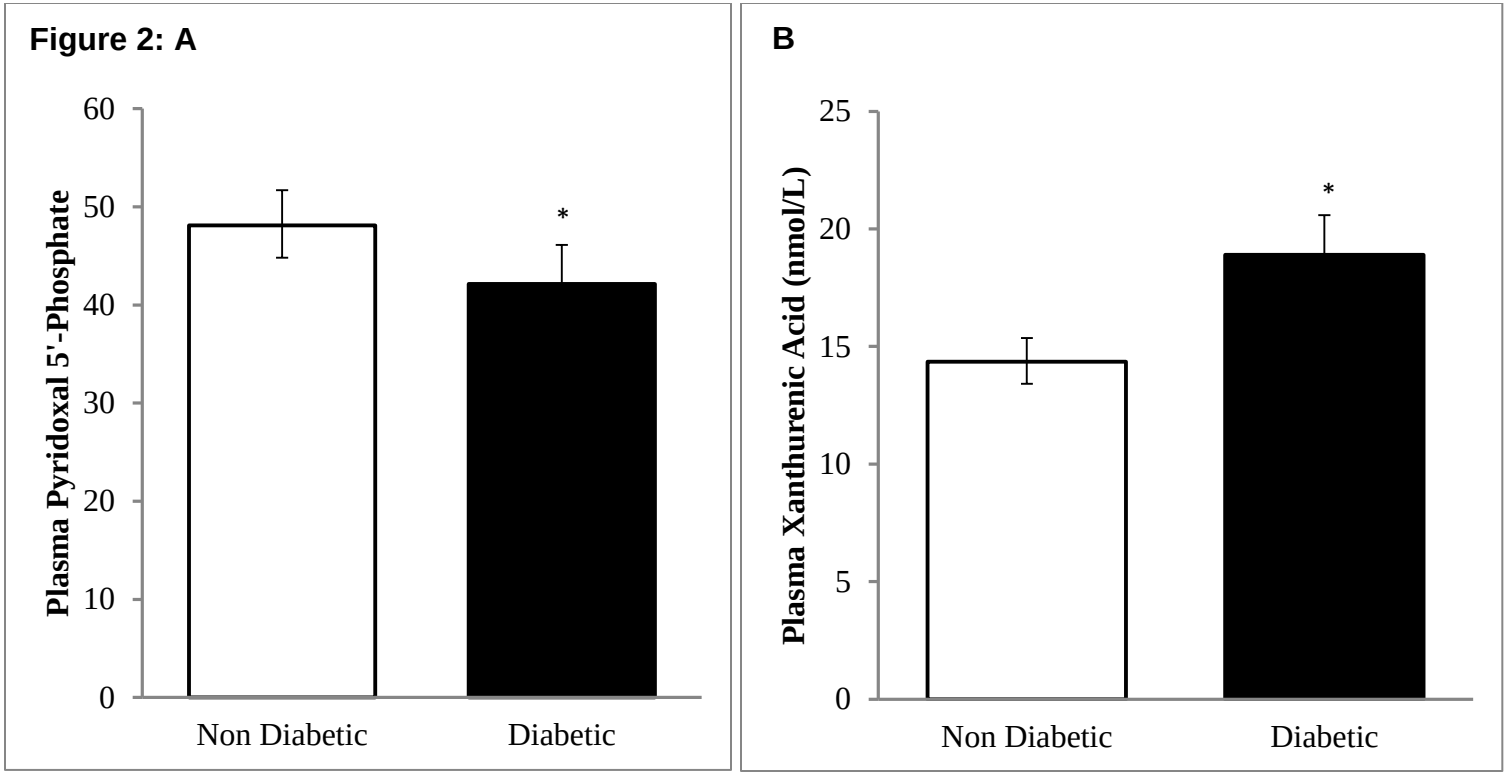

Figure 2: Average plasma concentration of A) pyridoxal-5'-phosphate (PLP) and B) xanthurenic acid (XA) amongst diabetics and non-diabetics in the Nutrition Aging and Memory in Elders study, adjusted for age and sex. Geometric means, presented in original scales.
* indicates difference compared to non-diabetic is statistically significant ($p<0.05$)

Table 4: HOMA2IR by plasma XA tertile and PLP sufficiency status[1]

XA Tertile	PLP Insufficient Mean(95%CI) HOMA2IR	PLP Sufficient Mean(95%CI) HOMA2IR	p-value*
1 (<11.2 nmol/L)	1.60 (1.29-1.91)	1.39 (1.17-1.61)	0.89
2 (11.2-18.1 nmol/L)	1.89 (1.57-2.22)	2.01 (1.79-2.22)**	0.99
3 (>18.1 nmol/L)	2.45 (2.06-2.84)**	1.72 (1.52-1.92)	0.02

[1]- Adjusted for age, sex, BMI, smoking status, NSAID use, vitamin B6 intake, total caloric intake, plasma folate, plasma homocysteine, plasma creatinine
* p-value for difference between tertiles
** $p < 0.05$ when compared to first tertile

Figure 3: Plasma XA, PLP, and Insulin Resistance among non-diabetics

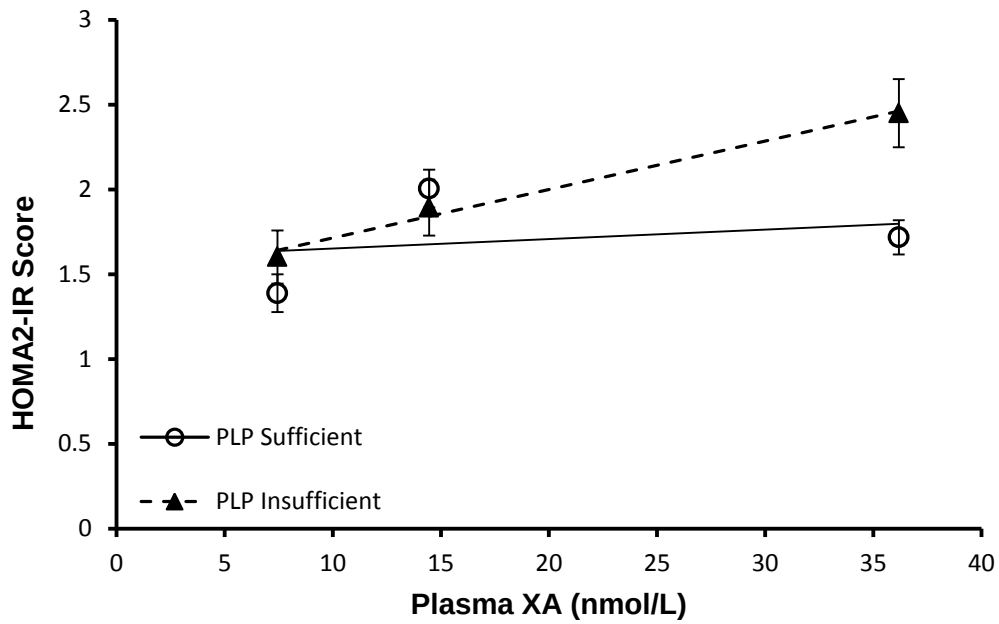

Figure 3: Non diabetics were categorized according to plasma PLP concentration (<30 nmol/L considered insufficient). Average concentration for plasma XA tertiles, presented in original scale, predict average HOMA2IR. Whiskers indicate 95% confidence interval, line indicates trendline. P-for trend PLP sufficient = 0.32, PLP insufficient = 0.002.

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Chapter 4

Association of vitamin B_6 nutritional status with inflammation and anemia in free-living Boston area elderly

Abstract

Conditions such as anemia, are commonly comorbid with inflammatory diseases. While several inflammatory diseases have been correlated with low plasma concentrations of the bioactive form of vitamin B6, pyridoxal 5'-phosphate (PLP), there has been little research into the health outcomes that may arise from unavailability of PLP for physiological processes such as heme production, contributing to risk of anemia. We examined the relation between plasma PLP, inflammatory marker C-reactive protein (CRP), and prevalence of anemia in the Nutrition Aging, and Memory in Elders cohort. Subjects in the lowest tertile category of plasma PLP had greater prevalence of anemia than the highest tertile category (34.9% vs 24.3%, $p=0.04$). The lowest tertile category of plasma PLP also had greater CRP concentrations than the second and third tertile categories, (10.8 vs 6.7 and 4.5 mg/L, respectively, $p<0.01$). The association between plasma PLP and anemia prevalence was modified by inflammation status; subjects with in the lowest tertile category of PLP and the highest plasma CRP concentration had the highest prevalence of anemia compared to subjects with moderate and low CRP concentrations (47.1% vs 24.1 and 21.1%, respectively $p<0.01$). Results suggest low plasma PLP and increased CRP are jointly related to increased prevalence of anemia. These data provide evidence for the possibility of other mechanisms of developing anemia during inflammation besides iron homeostasis. Future longitudinal studies are required to determine a causal relation between low plasma PLP and anemia.

Introduction

Anemia is common amongst elderly populations, and is comorbid with many inflammatory diseases [1, 2]. Often, inflammation is accompanied by subclinical nutrient deficiencies [3]. Specifically, several inflammatory diseases such as diabetes, cancer, cardiovascular disease, and rheumatoid arthritis (RA) are associated with low plasma concentrations of pyridoxal 5'-phosphate (PLP), the coenzyme form of vitamin B_6. [4-7]. To examine vitamin B6 nutritional status in rheumatoid arthritis patients, Chiang et al. administered tryptophan and methionine load tests, which measure xanthurenic acid and homocysteine excretion after being given a large oral dose of tryptophan and methionine. Chang et al. observed elevated xanthurenic acid and homocysteine excretion compared to controls, indicating low functional PLP status among RA patients [8]. Furthermore, PLP is more readily depleted from plasma and liver during periods of inflammation. This has been established from studies in a rat model of arthritis, which indicate that plasma and liver concentrations of PLP are lowered in response to induction of adjuvant arthritis, while no depletion was observed from muscle, PLP's main storage compartment [9]. While the fate of PLP during inflammation has not yet been determined, inflammation also increases tryptophan degradation via the kynurenine pathway and these kynurenine metabolites require PLP for their production [10].

Deficiency of PLP in plasma and liver stores may subsequently affect over 100 PLP-dependent reactions throughout the body [11]. In these reactions, PLP is required as cofactor for transamination, decarboxylation, and remethylation for important functions such as neurotransmitter synthesis, carbohydrate, lipid, and protein metabolism, and the synthesis of heme, the oxygen carrying component of hemoglobin [12]. Hemoglobin is the oxygen carrying protein found in red blood cells. Heme synthesis occurs mainly in

the bone marrow and liver, and PLP is a required cofactor for the first and rate-limiting enzyme in the synthesis of heme, δ-aminolevulinate synthase [13, 14].

We hypothesize that depletion of PLP from plasma and liver stores during inflammation may impair the function of δ-aminolevulinate synthase in heme synthesis, contributing to decreased production of hemoglobin, which may contribute, at least in part, to the development of anemia comorbid with inflammatory diseases.

We sought to determine the extent to which the association between plasma PLP concentration and inflammation affect prevalence of anemia. We investigated these associations within a community-based population of Boston area elders through use of cross sectional data from the Nutrition, Aging, and Memory in Elders (NAME) cohort.

Methods

Study Population

Data from the Nutrition, Aging, and Memory in Elders (NAME) study were used for this analysis and a detailed description of the study is provided elsewhere [15]. Briefly, participants aged 60 and over were recruited into NAME between 2003 and 2007 through Aging Services Access Points (ASAP), agencies that manage home care services for elders in Boston. To be eligible for ASAP, individuals had have annual income below $18,890, be residents of Boston, aged 60 and over, and require assistance with critical activities of daily living. Individuals from ASAP were excluded from NAME if they had severe vision or hearing impairment, were illiterate, did not speak English, refused or had a caregiver refuse participation, or if they currently have or previously had any of the following conditions: HIV/AIDS, epilepsy, schizophrenia, bipolar disorder, mental

retardation, or brain tumor. Informed consent was obtained from every participant and the NAME study protocol was approved by the Institutional Review Board at Tufts Medical Center.

Survey Measures:

Trained research staff conducted interviews over the course of 3 in-home visits for neuropsychological evaluation, general background including race (white non-Hispanic, black non-Hispanic, other), socioeconomic status, and health history questionnaires (including smoking status: current, former, never; current non-steroid anti-inflammatory (NSAID) use, and basic anthropometric measures including weight and height from which body mass index was calculated. Average daily vitamin B6 and iron intake were calculated from dietary assessment via semi-quantitative food frequency questionnaire [15-20].

One fasting blood sample was collected in K_2EDTA tubes and centrifuged in-home to obtain separated red blood cell and plasma samples. Samples were stored on ice during transport to the Nutrition Evaluation Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts within 2 hours of collection.

Samples were stored at -80°C until analysis of blood metabolites, B vitamins, and hematological measures.

Biochemical Measures:

Fasting blood draws were collected in K_2EDTA tubes, and centrifuged in-home with a portable centrifuge to obtain separated red blood cell and plasma samples. Samples were stored on ice during transport to the Nutrition Evaluation Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts within 2 hours of being drawn and stored at -80C until analyses.

Plasma PLP was measured using a tyrosine decarboxylase apoenzyme assay [21]. Hematologic measures, including red blood cell count and hemoglobin were measured using electronic impedance and light scatter (Penta 60C+, ABX Diagnostics). Anemia was defined according to World Health Organization (WHO) criteria, as having hemoglobin concentrations <13 g/dL for men and <12 g/dL for women [22]. Plasma CRP concentration was determined using the Immulite chemiluminescent assay [23].

Plasma folate and vitamin B_{12} were considered as potential confounders of the association between PLP and anemia, as they are interrelated with PLP in one-carbon metabolism and are also risk factors for anemia. Low plasma folate is a risk factor for megaloblastic anemia and low plasma vitamin B_{12} for pernicious anemia. Plasma folate and vitamin B_{12} were measured using Immulite chemiluminescent assay (Diagnostic Products/Siemens). Plasma homocysteine and creatinine were also included as covariates in the model for their participation in one-carbon metabolism. Plasma total homocysteine was measured by HPLC [24]. Plasma creatinine was measured using a Roche Cobas Mira (F. Hoffmann-La Roche Ltd, Basel Switzerland) and an Olympus AU 400e (Olympus America Inc., Center Valley, PA, USA) [25].

Of the original 1,259 subjects enrolled in NAME, 913 subjects had complete data with respect to exposures, outcomes, and covariates for the present analysis. (Figure 1)

Covariates:

Covariate data was collected during the in-home visit as part of health status and food frequency questionnaires. Covariates included age; sex; BMI calculated from data collected on weight and height; race/ethnicity categorized as white non-Hispanic, black non-Hispanic and other; smoking status categorized as current, previous, or never; NSAID use categorized as users or non-users; diabetes history categorized as diabetics or non-diabetics; and hypertension categorized as hypertensive (systolic blood pressure >140/diastolic blood pressure > 90) or normotensive (SBP ≤140 and DBP ≤90; and vitamin B-6 and iron intakes.

Statistical Analysis:

Data were analyzed using SAS v. 9.3 (Cary, NC). Statistical significance refers to $P<0.05$. Plasma PLP was grouped into tertile categories for descriptive analysis. Analysis of variance was used to compare unadjusted mean for continuous subject characteristics and Pearson's chi square test of independence was used to compare unadjusted percentages for dichotomous subject characteristics across tertile categories of plasma PLP concentration. Where necessary, variables with skewed distributions were log-transformed to meet assumptions for statistical analysis. In such cases, formal analysis was conducted on log-transformed data, and geometric means are presented in the data's original scale.

To examine the association between PLP and inflammation, geometric mean PLP concentrations were compared across three categories of CRP, low (<3 mg/L), moderate (3-10 mg/L), and high (>10 mg/L) using analysis of covariance (ANCOVA) with Tukey's Honestly Significant Difference to adjust for multiple comparisons. Covariates described above were included in the ANCOVA model to adjust the geometric mean PLP concentrations for potential confounding variables

ANCOVA models were also used to examine the relationship between plasma PLP concentrations and anemia status, coded as 1 for subjects meeting the WHO definition as described above and 0 for those who did not meet the WHO definition. For these analyses, prevalence of anemia was compared across tertile categories of plasma PLP concentrations adjusting for covariates as described above and using ANCOVA with Tukey's Honestly Significant Difference to account for multiple comparisons.

To test the hypothesis that the prevalence of anemia would be greater among individuals with inflammation in the presence of low PLP, we performed an ANCOVA with Tukey's Honestly Significant Difference to adjust for multiple comparisons, modelling anemia as the outcome and including both plasma CRP as categorized above and PLP as tertile categories, the cross-product term to test for the interaction of these two factors, and covariates listed above to account for potential confounding. Based on a statistically significant cross-product term, the association of PLP and anemia was stratified by CRP category to examine the nature of the interaction between CRP and PLP on anemia prevalence.

Logistic regression was used to analyze the association between kynurenine metabolites and odds of anemia, after adjusting for age, sex, race, plasma folate, plasma vitamin B12, smoking status and iron intake.

Results

The tertile categories of plasma PLP concentration were as follows: Tertile Category 1: 4.3-31.4 nmol/L, Tertile Category 2: 31.5-65.6 nmol/L and Tertile Category 3: 65.8-857.8 nmol/L. Coincidentally, the maximum plasma PLP concentration of tertile 1 is approximately the cutoff for PLP insufficiency, therefore tertile category 1 encompasses subjects with both deficient and low-normal plasma PLP concentrations [26]. Across tertile categories of plasma PLP concentration, higher plasma PLP was associated with higher vitamin B-6 intake, higher iron intake, higher plasma folate concentration, and higher vitamin B-12 concentration. Higher plasma PLP concentrations were also associated with lower BMI, lower plasma homocysteine concentration, lower prevalence of current smokers, lower prevalence of non-Hispanic black race/ethnicity, and lower prevalence of diabetes. The percent of women, hemoglobin concentration, plasma creatinine concentration, and prevalence of current NSAID use did not differ across PLP tertile categories (Table 1).

The proportion of subjects with plasma PLP indicating PLP deficiency (PLP < 20 nmol/L, [27]) was 15.0% (n=137). Among these PLP deficient subjects, only 65.7% (n=90) had vitamin B-6 intake below Recommended Daily Allowance (<1.5 mg/day for women and 1.7 mg/day for men) and 18.6% (n=25) did not meet Estimated Average

Requirements for their gender and age (1.3 mg/day for women and 1.4 mg/day for men) (data not shown) [28].

To examine the relation between PLP and inflammation, we compared geometric mean plasma PLP concentrations for low, moderate, and high plasma CRP concentrations in Figure 2. Subjects with in the lowest PLP tertile category had the highest concentration of CRP (10.81 mg/L (95% CI: 9.5-12.2), compared to the second (6.70 mg/L (95% CI: 5.4-7.9) $p<0.0001$) and third tertile categories (4.51 nmol/L (95% CI: 3.2-5.9), $p<0.0001$) after adjusting for age, sex BMI, smoking status, NSAID use, vitamin B-6 intake, and plasma folate, vitamin B-12, homocysteine and creatinine concentrations.

Examination of the prevalence of anemia by tertile category of plasma PLP concentration (Figure 3), demonstrated that subjects in the lowest tertile category of plasma PLP status had the highest prevalence of anemia (34.9% (95%CI: 29.4-40.3) after adjusting for age, sex, smoking status, race, vitamin B-6 intake, iron intake, and plasma folate and vitamin B-12 concentrations, which was not significantly different from anemia prevalence in the second tertile (26.5% (95%CI: 21.4-31.5), $p=0.07$) but was significantly higher than the third tertile (24.28%, (95%CI: 18.8-29.7) $p=0.04$).

To further investigate the combined relationship of inflammation and plasma PLP on anemia prevalence, we tested for the presence of an interaction by including both PLP tertile and CRP categories and their cross-product term, and found the interaction between the two was statistically significant ($p=0.02$); consequently we stratified the association between anemia prevalence and plasma PLP by CRP category (Figure 4). For subjects in the two higher tertile categories of plasma PLP concentrations, there was no

difference in anemia prevalence between subjects with low, medium, or high CRP concentrations. However, among subjects in the lowest PLP tertile category, prevalence of anemia was 47.1% in those with high CRP concentrations, statistically significantly higher ($P<0.001$) that the prevalence of 24.7% and 21.1% in those with moderate and low CRP concentrations, respectively. This indicates that high inflammation was an additional contributor to anemia risk only among subjects with low plasma PLP concentrations.

None of the kynurenine metabolites measured were associated with increased odds of anemia (Table 2).

Discussion

The present study finds inflammation as indicated by the plasma the prevalence of anemia depends on both plasma PLP concentration and presence of inflammation. An increase in prevalence of anemia was observed among subjects with insufficient PLP only with high levels of inflammation were present.

We also confirmed the previously reported inverse association between plasma PLP concentration and inflammation. These earlier studies demonstrated that low plasma PLP was associated with elevated inflammatory markers and flare ups of increased disease severity [4, 8, 9, 29]. These studies suggest that mechanisms leading to lower plasma PLP are variable and may be related to current inflammatory state, rather than a static effect of disease status. PLP distribution is decreased mainly in liver and plasma of rats induced with RA, indicating PLP depletion during inflammation is tissue specific [9]. Heme biosynthesis also occurs in liver as well as bone marrow, and PLP is required as a

cofactor for δ-aminolevulinate synthase, the enzyme in the first and rate-limiting step of heme biosynthesis. Thus, it stands to reason that this inflammation-associated PLP depletion in locations of heme synthesis may result in anemia. Our findings that the combination of high inflammation and low plasma PLP is associated with the highest prevalence of anemia suggest that production of heme, and subsequently the production of fully functional red blood cells, may be affected by inflammation-related PLP depletion, and not necessarily a result of inflammation alone. Future studies may establish this as a mechanism other than iron homeostasis [30] that may contribute to the development of anemia in a heightened inflammatory state.

The current body of evidence has not found consistent associations between PLP status and anemia. While Huang et al. observed no correlation between PLP and hemoglobin after vitamin B6 supplementation, Cohick and Bhattacharjee present a case study of an anemic RA patient for whom vitamin B6 supplementation increased hemoglobin and plasma ferritin concentrations [31, 32]. The findings presented here support some of the observations of Cohick and Bhattacharjee; however, serum ferritin was not assessed for our cohort. Further research on the production of functional red blood cells during periods of inflammation and PLP deficiency must be done to strengthen the connection of our findings to the functionality of δ-aminolevulinate synthase, and the development of anemia independent of the actions of iron status.

There are limitations to this study that preclude definitive conclusion that PLP is causally related to anemia. Anemia is most commonly known to arise from aberrant iron homeostasis. Hepcidin, a hormone that regulates iron, is induced during inflammation and hepcidin concentration could be confounding the results presented here [33, 34]. Due

to limitations of the dataset collected from NAME, the contribution of hepcidin action on hemoglobin concentrations cannot be accounted for in the present study; however, the associations between PLP, inflammation and anemia presented here were found to be independent of iron intake. Additionally, due to the cross sectional nature of this analysis, we are unable to establish a temporal relation between vitamin B-6 status, inflammation, and anemia.

Nationally, the prevalence of anemia in older Americans (aged 50+) is estimated to be 20% [1], and this is elevated to between 30 and 60% in RA patients [2, 35]. Low-grade inflammation similar to that seen in RA is associated with high anemia prevalence and low hemoglobin concentrations in both diseased and non-diseased populations [36, 37].The data presented here indicate that anemia prevalence is inversely related to vitamin B6 nutrient status, as assessed here by plasma PLP. Furthermore we found the effects of PLP insufficiency on anemia were exacerbated by increased inflammation, since the subset of our cohort with both these features had the highest prevalence of anemia. The findings suggest that elevated anemia prevalence comorbid with inflammatory conditions may be addressed by either attenuating inflammation or improving vitamin B6 nutritional status in the population.

Figures and Tables

Figure 1:

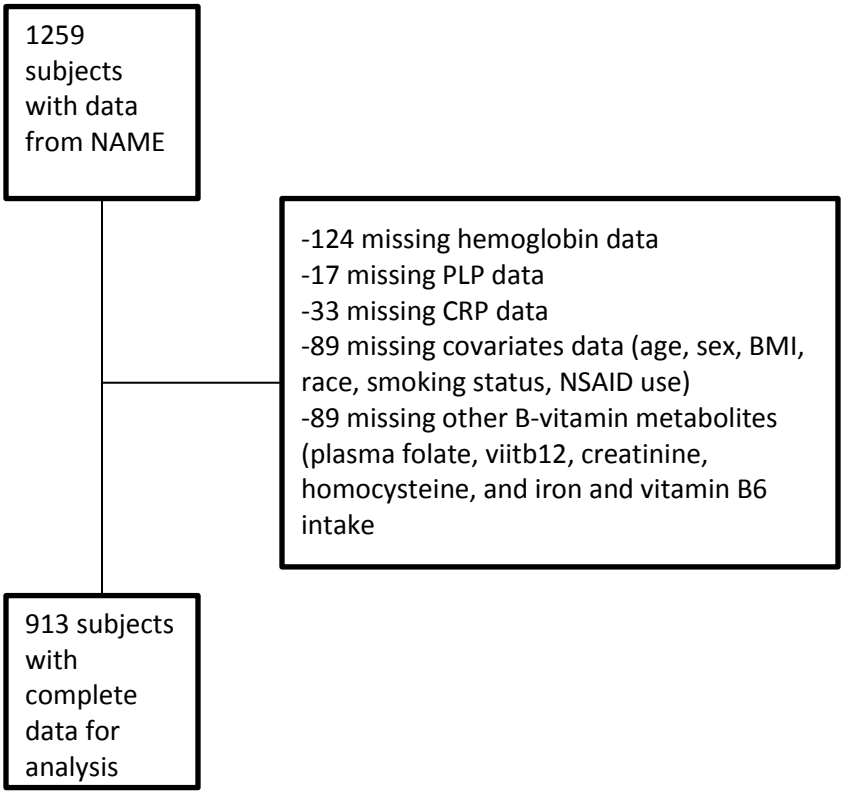

Figure 1: Schema of subjects from the Nutrition, Aging, and Memory in Elders study with data available for analysis.

Table 1. General characteristics of the Nutrition, Aging, and Memory in Elders cohort, by plasma PLP tertile category

	1 (n=299)	2 (n=303)	3 (n=311)	
Characteristic	Mean ± SD	Mean ± SD	Mean ± SD	P-value*
General characteristics				
Age (yrs)	74.14 ± 8.06	75.79 ± 8.62	75.81 ± 8.52	0.0200
Female (%)	233 (77.93)	233 (76.90)	237 (76.21)	0.8792
BMI (kg/m^2)	33.13 ± 9.70	31.77 ± 9.00	30.13 ± 6.73	<.0001
Hemoglobin (mg/dL)	12.99 ± 1.66	13.19 ± 1.50	13.06 ± 1.48	0.2492
Non-Hispanic Black (%)	131 (43.81)	112 (36.96)	82 (26.37)	0.0001
Non-Hispanic White (%)	160 (53.51)	187 (61.72)	219 (70.42)	0.0001
Other race (%)	8 (2.68)	4 (1.32)	10 (3.22)	0.0001
Current smoker (%)	76 (25.42)	47 (15.51)	26 (8.36)	<.0001
NSAID use (%)	37 (12.37)	45 (14.85)	43 (13.83)	0.6741
Plasma measures				
Pyridoxal Phosphate (nmol/L)	20.50 ± 6.19	45.39 ± 9.47	141.70 ± 97.38	<.0001
Plasma folate (ng/mL)	10.04 ± 5.29	14.31 ± 7.36	20.26 ± 9.26	<.0001
Vitamin B-12 (pg/mL)	542.76 ± 760.52	515.90 ± 217.34	721.41 ± 456.77	<.0001
Homocysteine (μmol/L)	12.79 ± 5.43	11.79 ± 5.81	11.22 ± 4.23	<.0001
C-reactive protein (mg/L)	10.75 ± 15.38	6.58 ± 9.46	4.66 ± 6.73	<.0001
Creatinine[1]	1.04 ± 0.69	1.08 ± 1.09	1.11 ± 1.08	0.6332
Nutrient intakes				
Vitamin B-6 intake (mg)	4.12 ± 18.40	4.48 ± 8.25	12.57 ± 25.23	<.0001
Iron intake (mg)	16.17 ± 12.10	19.74 ± 14.22	22.22 ± 17.23	<.0001

[1] – Standardized to IDMS standard

Figure 2

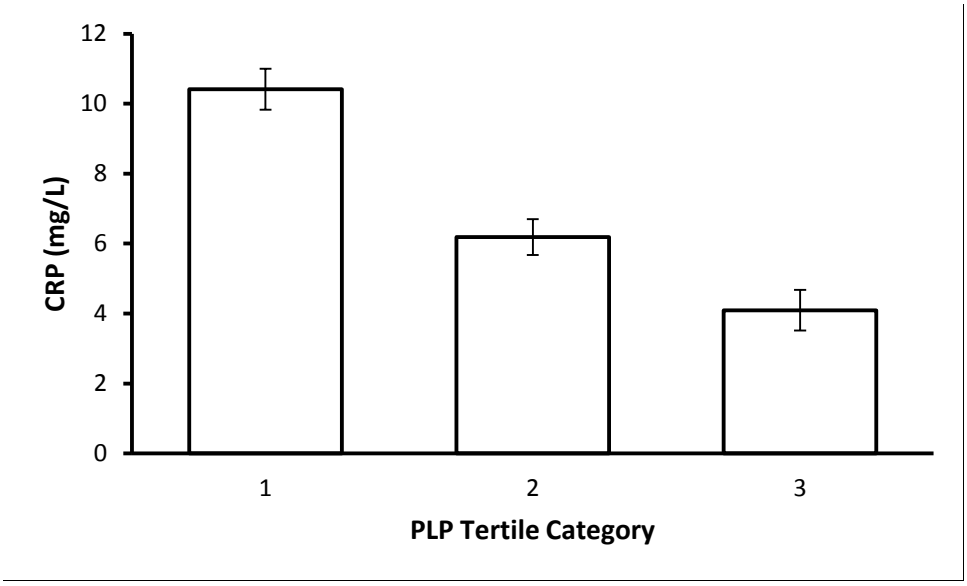

Figure 2: Geometric mean CRP concentrations for subjects in PLP tertile categories (PLP Tertile 1: 4.3-31.4 nmol/L, Tertile 2: 31.5-65.6 nmol/L and Tertile 3: 65.8-857.8/ nmol/L), after adjustment for age, sex, BMI, smoking status, NSAID use, vitamin B-6 intake, and plasma folate, homocysteine, creatinine, and vitamin B-12 concentrations. Boxes indicate mean concentration; whiskers indicate standard error of the estimate.

Figure 3

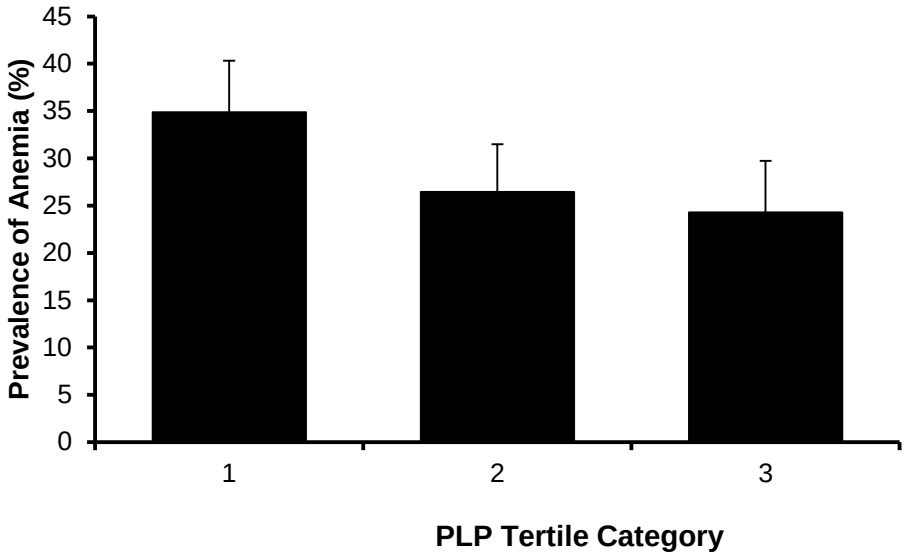

Figure 3: Estimated prevalence of anemia for subjects in each tertile of plasma PLP concentration (PLP Tertile 1: 4.3-31.4 nmol/L, Tertile 2: 31.5-65.6 nmol/L and Tertile 3: 65.8-857.8/ nmol/L) after adjusting for age, sex, smoking status, race, vitamin B-6 intake, iron intake, and plasma folate and vitamin B-12 concentrations. Prevalence was calculated using analysis of covariance (ANCOVA) with Tukey's Honestly Significant Difference to adjust for multiple comparisons. Boxes indicate estimated prevalence; whiskers indicate standard error of the estimate.

Figure 4

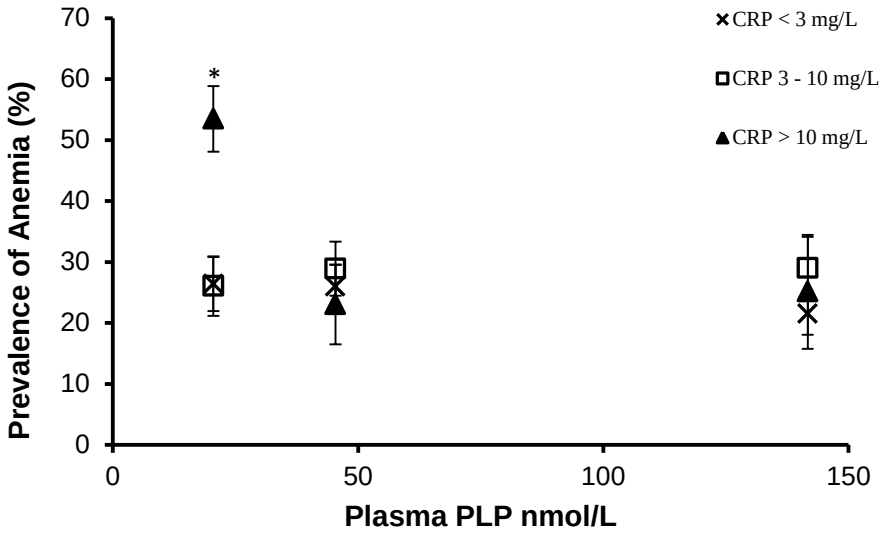

Figure 4: Anemia prevalence by plasma PLP tertile and CRP concentration Points are positioned at median value for plasma PLP for the first, second, and third tertiles (PLP Tertile 1: 4.3-31.4 nmol/L, Tertile 2: 31.5-65.6 nmol/L and Tertile 3: 65.8-857.8/ nmol/L). Estimated prevalence of anemia for subjects in each tertile of plasma PLP concentration after stratifying by low (<3 mg/L, filled circles) moderate (3-10 mg/L, empty squares) and high (>10 mg/L, filled triangles) plasma CRP concentrations. Prevalence was calculated using analysis of covariance (ANCOVA) with Tukey's Honestly Significant Difference to adjust for multiple comparisons, adjusted for age, sex, race, smoking status, vitamin B-6 and iron intake, and plasma folate and vitamin B-12 concentrations. * -indicates significant difference between both other CRP concentrations within that PLP tertile ($p<0.05$).

Table 2: Association of kynurenine metabolites with odds of anemia

Metabolite	OR	95 % CI
Anthranilic Acid	1.00	0.99-1.01
Kynurenic Acid	1.00	0.99-1.00
Hydroxykynurenine	1.00	1.00-1.00
Hydroxyanthranilic acid	1.00	1.00-1.00
Xanthurenic Acid	0.99	0.98-1.01
Kynurenine	1.12	0.87-1.45
Tryptophan	0.99	0.99-1.00

Logistic Regression adjusted for age, sex, race, smoking status, plasma folate, plasma vitamin B12, and iron intake.

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Chapter 5

Association between pyridoxal-5'-phosphate and kynurenine metabolites, with cognitive impairment and depression in elderly adults

Abstract

Inflammatory diseases are often accompanied by comorbid conditions such as depression. While it has long been observed that low plasma PLP concentrations are associated inflammatory diseases and increased markers of inflammation, there is little known about the ramifications of low plasma PLP concentrations on PLP-dependent functions in the body. Importantly, DOPA-decarboxylase, an enzyme required for neurotransmitter production, requires PLP as a cofactor. Inflammation is also associated with increased tryptophan degradation to kynurenine metabolites that may mitigate tissue damage due to immune responses. Enzymes that produce many kynurenine metabolites require PLP. We hypothesize increased PLP utilization in tryptophan degradation during inflammation may underlie the reduction in plasma PLP and subsequent PLP insufficiency affects neurotransmitter synthesis, contributing to risk of cognitive impairment or depression. We examined the relation between PLP, kynurenine metabolites, and measures of cognitive function and depression by analyzing data and measuring kynurenine metabolites using LCMS/MS in plasma samples from the Nutrition Aging and Memory in Elders Study. Factor scores for attention were 0.07 points greater for between 25th and 75th percentiles of plasma PLP concentration ($p=0.02$). Similarly, Mini Mental State Examination scores were 0.22 points greater, indicating fewer symptoms of cognitive impairment ($p=0.03$). Associations between kynurenine metabolites and cognitive impairment and depression failed to meet statistical significance. Results suggest that plasma PLP is related to cognitive measures, however we did not observe evidence to suggest that increased kynurenine metabolism underlies this association.

Background

Depression is often observed comorbid with other medical conditions, including those characterized by inflammation such as diabetes, RA and CVD [1-3]. It has been well documented that these inflammatory conditions are associated with low circulating levels pyridoxal-5'-phosphate (PLP), a bioactive form of vitamin B6 [4]. Low PLP is correlated with markers of high inflammation [5, 6] and increased disease severity [7].

While PLP deficiency due to diet is rare in the United States, [8], low plasma PLP concentrations, such as those observed during inflammation [5, 6] may impair functions that require PLP for biochemical reactions. PLP is directly involved in neurotransmitter synthesis as a required cofactor for the enzyme DOPA decarboxylase, which converts the neurotransmitter precursor L-3,4-dihydroxyphenylalanine (L- DOPA) to dopamine [9]. Additionally, experimental studies in Sprague-Dawley rats have demonstrated the effect of PLP deficiency on brain function, as rats on pyridoxine deficient diets had reduced glucose utilization in basal ganglia, hypothalamus, limbic, and sensory motor systems of the brain [10]. Low plasma PLP during inflammation may impair neurotransmitter synthesis due to insufficient PLP for DOPA decarboxylase function, or aberrant glucose utilization in the brain similar to that observed in rats.

During inflammation, there is also increased degradation of tryptophan through the kynurenine pathway due to induction of indoleamine 2,3 dioxygenase (IDO), the first enzyme in the kynurenine pathway in extrahepatic tissues. Inflammatory cytokines that are upregulated during inflammation, such as interferon gamma, induce IDO [11] and increase kynurenine production. The enzymes that further metabolize kynurenine,

kynurenine aminotransferase (KAT) and kynureninase require PLP as a cofactor to produce kynurenine metabolites. KAT catalyzes the conversions of kynurenine to kynurenic acid (KA) and 3-hydroxykynurenine (HK) to xanthurenic acid (XA) [12]; whereas kynureninase catalyzes the conversions of kynurenine to anthranilic acid (AA) and 3-hydroxykynurenine to 3-hydroxyanthranilic acid (HAA) [13] (Figure 1). These metabolites have been demonstrated to have immunomodulatory properties, which are important in limiting the immune response [14-16]. Increased kynurenine metabolism may underlie the low plasma PLP observed during inflammation.

Increased kynurenine pathway activation during inflammation may increase PLP utilization by KAT and kynureninase, resulting in increased kynurenine metabolite concentrations and reduced plasma PLP concentrations. Based on this available information, we hypothesize that low plasma PLP status during chronic inflammation may affect neuropsychological function through the role of PLP in neurotransmitter synthesis. To investigate this hypothesis, we examined the relationships of plasma PLP and kynurenine metabolites with cognition and depression in elderly community-based, home-bound Boston residents.

Methods

Study Population

Data and plasma samples collected in 2003-2007 from the Nutrition Aging and Memory in Elders study were used for this analysis [17]. Participants aged 60 and over were recruited into NAME through Aging Services Access Points (ASAP), agencies that

managed home care services for elders from Boston. To be eligible for ASAP, individuals had to be low-income (<$18,890) residents of Boston, aged 60 and over, and required assistance with critical activities of daily living. Individuals from ASAP were excluded from NAME if they were severely vision or hearing impaired, illiterate, refused or had a caregiver refuse participation, did not speak English, or if they ever had any of the following conditions: HIV/AIDS, epilepsy, schizophrenia, bipolar disorder, mental retardation, or brain tumor. Informed consent was obtained from every participant and the NAME study protocol was approved by the Institutional Review Board at Tufts Medical Center.

Trained research staff conducted interviews and took anthropomorphic measures over 3 in-home visits. Information collected included age, education, occupation, family size, migration and acculturation, household income, sources of social support, food security, a detailed neuropsychological evaluation, health history questionnaires, and dietary assessment via semi-quantitative food frequency questionnaire [17-22]. One fasting blood draw was also conducted in-home. Fasting blood samples were collected in K_2EDTA tubes, and centrifuged in-home to obtain separated red blood cell and plasma samples. Samples were stored on ice during transport to the Nutrition Evaluation Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts within 2 hours of collection.

Samples were stored at -80°C until analysis of blood metabolites, B vitamins, and hematological measures.

Laboratory Analyses

Plasma Kynurenine Metabolites: Plasma tryptophan metabolites (kynurenic acid, kynurenine, anthranilic acid, 3-hydoxykynurenine 3-hydroxyanthrnailic acid, anthranilic acid, tryptophan, xanthurenic acid) were measured simultaneously using the methods described by Midttun et al. [23]. Briefly, thawed plasma samples were deprotonated using a 1:1 ratio of plasma to 6% (w/v) trichloroacetic acid that was spiked with 100 nmol/mL deuterated xanthurenic acid standard (US Biological, Salem, MA). The mixture was immediately vortexed, and shaken at 4°C for 1 hour. Samples were then centrifuged at 13,000 rpm for 30 minutes. The supernatant was filtered through 0.22 µm Durapore® filter plates (Milipore, Cork, IRL) and sample was loaded into glass vial inserts for LC/MS-MS analysis. An Agilent 1300 series HPLC system was equipped with an in-line filter (2 mm, 0.2 µm, 70x 0.12mm) and a Zorbax Eclipse XDB-C8 Rapid Resolution HT reversed-phase C-8 column (2.1 x 50 mm, 1. 8 µm) with Poroshell 120EC-C8 2.1 mm ultra-high pressure liquid chromatography guard column. The column was kept in a thermostated column compartment set to 40°C. The HPLC system was connected to an AB Sciex 5500 linear ion trap tandem mass spectrometer with TurboSpray ion source. The mobile phase solutions were A: 650 mM acetic acid, B: 100 mM heptafluorobutyric acid, and C: 90% (v/v) acetonitrile in water. A thermostated autosampler compartment kept samples at 4°C. Injection volume was 5 µL. Mobile phase solutions were delivered through the system at a constant flow rate of 0.4 mL/minute in the following ratios at the designated time points: 0 minutes: 98% A, 2% B; 2.2 minutes: 78% A, 2% B 20% C; 2.3 minutes: 60%A, 2% B, 38% C; 3.3 minutes: 40% A, 2%B, 58% C; 3.9 minutes: 2%A, 2%B, 96% C; 4 minutes: 98% A, 2% B; 6 minutes: 98%A,

2%B. The total run time was 6.5 minutes. The mass spectrometer was set to positive polarity in multiple reaction monitoring mode, using an ion source temperature of 550°C and voltage of 5500V.

Plasma PLP: PLP was measured using a tyrosine decarboxylase apoenzyme assay [24]. For some analyses, subjects were grouped based on plasma PLP sufficiency: PLP insufficient (<30 nmol/L) and PLP sufficient (≥ 30 nmol/L). We used a previously established cutoff [25] of < 30 nmol/L to define PLP insufficiency.

Other Laboratory Analyses: Plasma concentrations of folate and vitamin B12 were measured using Immulite Chemiluminescent Assay, and total homocysteine was measured using HPLC [26]. Plasma creatinine was measured using a Roche Cobas Mira (F. Hoffmann-La Roche Ltd, Basel Switzerland) and an Olympus AU 400e (Olympus America Inc., Center Valley, PA, USA) [27]. ApoE4 genotype was determined using PCR and gel electrophoresis [28].

Dietary Assessment

Dietary intakes were assessed using the Harvard semi-quantitative food frequency questionnaire (FFQ) [18-22]. The FFQ consists of a list of foods with a standard serving size and a selection of 9 frequency categories ranging from never or <1 serving/month to ≥ 6 servings/day. The FFQ was interviewer-administered in subjects' homes, which allowed specific details about food items and vitamin supplements to be obtained and confirmed.

Mental and psychological outcome measures:

NAME had several outcome measures related to depression and cognition including Center for Epidemiological Studies Depression Scale (CES-D), Mini-Mental State Examination (MMSE), and a battery of cognitive tests used to develop factor scores for three domains of cognition: memory, attention and executive function [17]. During in-home visits, interviewers administered MMSE as a measure of cognitive impairment. MMSE scores range from 0 to 30, with higher scores indicating less symptoms of cognitive impairment [29]. Per protocol of the parent study, subjects with MMSE scores 10 or below were excluded from NAME [17]. Additionally, subjects with IQ less than 75, as assessed by the North American Adult Reading Test, were also excluded. CES-D was used as a measure of depressive symptoms [30]. CES-D scores range from 0 to 60. A depression variable was created using CES-D score indicating "Depressed" if CES-D score greater than 16, indicating a subthreshold for , otherwise subjects were categorized as "Not Depressed".

Health and Education History

Data on age, sex, height, weight, smoking status, smoking status (never, former, current), alcohol drinking status, and highest education level were collected as part of health history questionnaire during in-home interview. BMI was calculated as height in meters divided by the square of weight in kilograms [17].

Covariates:

A number of variables were considered as potential confounders of the associations examined. These included age, sex, BMI, smoking status (never, former, current), alcohol

drinking status, highest education level, hypertension, ApoE4 genotype, plasma folate, plasma total homocysteine, plasma creatinine, and dietary intakes of vitamins B6 and vitamin B12 and energy.

Statistical Methods:

Tobit regression was used to analyze MMSE scores to account for ceiling effects at the upper bound of MMSE score = 30. Multiple linear regression was used to analyze factor scores for memory, cognition, and executive function predicted by plasma PLP concentration or kynurenine metabolite concentration. For multiple linear regression analysis, skewed independent variables were scaled to calculate the difference in dependent variable between subjects in the 25th vs the 75th percentile of the independent variable, difference in IQR. Logistic regression was used to predict depressive symptoms as indicated by CES-D score. Regression analyses were adjusted for covariates age, gender, BMI, highest education obtained, smoking status, alcoholic drinking status, ApoE4 genotype, hypertension, plasma folate, plasma vitamin B12, plasma homocysteine, and plasma creatinine concentration, race, and diabetes status.

Results

On average, participants in the NAME cohort were approximately 75 years old, obese with a BMI of 31.6, and the majority of participants were women (76.72%) (Table 1). Approximately one third of participants had diabetes (36.56%) and approximately one third had plasma PLP below 30 nmol/L) (31.91%). The majority of participants were non-Hispanic white (61.97%) and approximately one third of participants did not complete high school education (33.44%).

Higher PLP concentration was associated with increased attention in the multivariate analysis, indicating the difference in factor score for attention between a typically “low” and typically “high” plasma PLP concentration is an increase of approximately 0.07 points (β=0.07 p=0.02) (Table 2). Mini Mental State Examination score also increased with plasma PLP concentration with the change in MMSE score of 0.22 per change IQR of plasma PLP. Factor score for executive function was similarly trending towards significant, and memory was not associated with plasma PLP concentrations.

The regression coefficients representing the association between kynurenine metabolites and cognition are displayed in Tables 3 through 6. When calculated as difference in cognition factor scores per change in IQR, none of the associations with kynurenine metabolites met statistical significance. Changes in factor scores for memory with KA and HAA concentrations were trending towards significance (AA: 0.07, p=0.09; KA: -0.09, p=0.09) as was the change in executive function per IQR KA (0.08, p=0.10); but they represent such small changes in factor scores across drastic differences in kynurenine metabolite concentrations they may not be physiologically relevant.

Additionally, none of the kynurenine metabolites were significantly associated with MMSE (Table 6) or depression as assessed by CES-D (Table 7).

Discussion

We observed that plasma PLP was positively associated with the factor score for attention, indicating higher plasma PLP concentrations are associated with increased attentiveness, an association previously reported by Moorthy et al. in a pooled dataset

combining data from both NAME and the Boston – Puerto Rico Health Study [31]. Here we present the findings from NAME alone.

The conversion of L-dihydroxyphenylalanine (L-DOPA) to dopamine and serotonin via DOPA decarboxylase requires PLP as a cofactor. Healthy stores of plasma PLP may allow this reaction to produce dopamine at sufficient rate for conversion to other neurotransmitters such as epinephrine and norepinephrine, involved in fight or flight reactions, among others. There have not been many studies examining the specific associations of cognition with plasma PLP rather than B-vitamin supplementation [32, 33]. A few studies have examined the association of PLP with L-DOPA in the context of Parkinson's Disease, for which L-DOPA is first-line treatment [34, 35]. Parkinson's disease patients treated L-DOPA had lower plasma PLP concentrations and increased homocysteine levels than patients not taking L-DOPA, suggesting increased B-vitamin requirements to metabolize L-DOPA [34]. Tan et al. found the less active polymorphism for catechol-O-methyltransferase (COMT), an enzyme downstream of DOPA decarboxylase that produces dopamine from L-DOPA, experienced the greatest benefit of pyridoxine supplementation with respect to improved motor function and activities of daily living [35]. Such findings suggest that L-DOPA metabolism and plasma PLP concentrations are connected, though it would be hard to extrapolate such effects to Parkinson's Disease-free population. The effects of any singular neurotransmitter would be hard to discern when considering the role of PLP in L-DOPA metabolism, but sufficient PLP concentrations for L-DOPA metabolism may be beneficial in maintaining a healthy neurochemical balance.

In the present study we observed very few associations observed between kynurenine metabolites and measures of cognition or depression. Data from prior studies do not agree with our own failure to see associations between cognition or depression with KA [36 , 37]. Previous research of KA on cognition and depression observed that inhibition of KAT in rats reduce KA in brain and appeared to improve cognition [38]. Low KA concentrations were also observed in subjects with depressive symptoms and suicidality [39].

Given the fact that NAME study excluded subjects with severe cognitive impairment (MMSE scores below 9), it is possible that the associations between kynurenine metabolites and cognition are only observed in the most extreme cases. This study examined circulating concentrations of kynurenine metabolites, which are relatively easy to collect and may offer insight into the perturbations to the kynurenine pathway during disease, but may not offer a complete picture with respect to the functions of kynurenine metabolites within the brain or nervous system. Additionally, the cross-sectional nature of the NAME study design precludes us from observing any temporal relation between metabolites and cognition that may occur.

In conclusion, this study found that plasma PLP is associated with the attention component of cognition, and we did not observe any relations between kynurenine metabolites and other cognitive domains, cognitive impairment as measured by the MMSE, or depression. Future studies containing longitudinal design are expected to more clearly elucidate the relationship between kynurenines and cognition. Further research into the role of PLP in the production of neurotransmitters may also allow us to better understand the development and prevention of cognitive impairment.

Figures and Tables

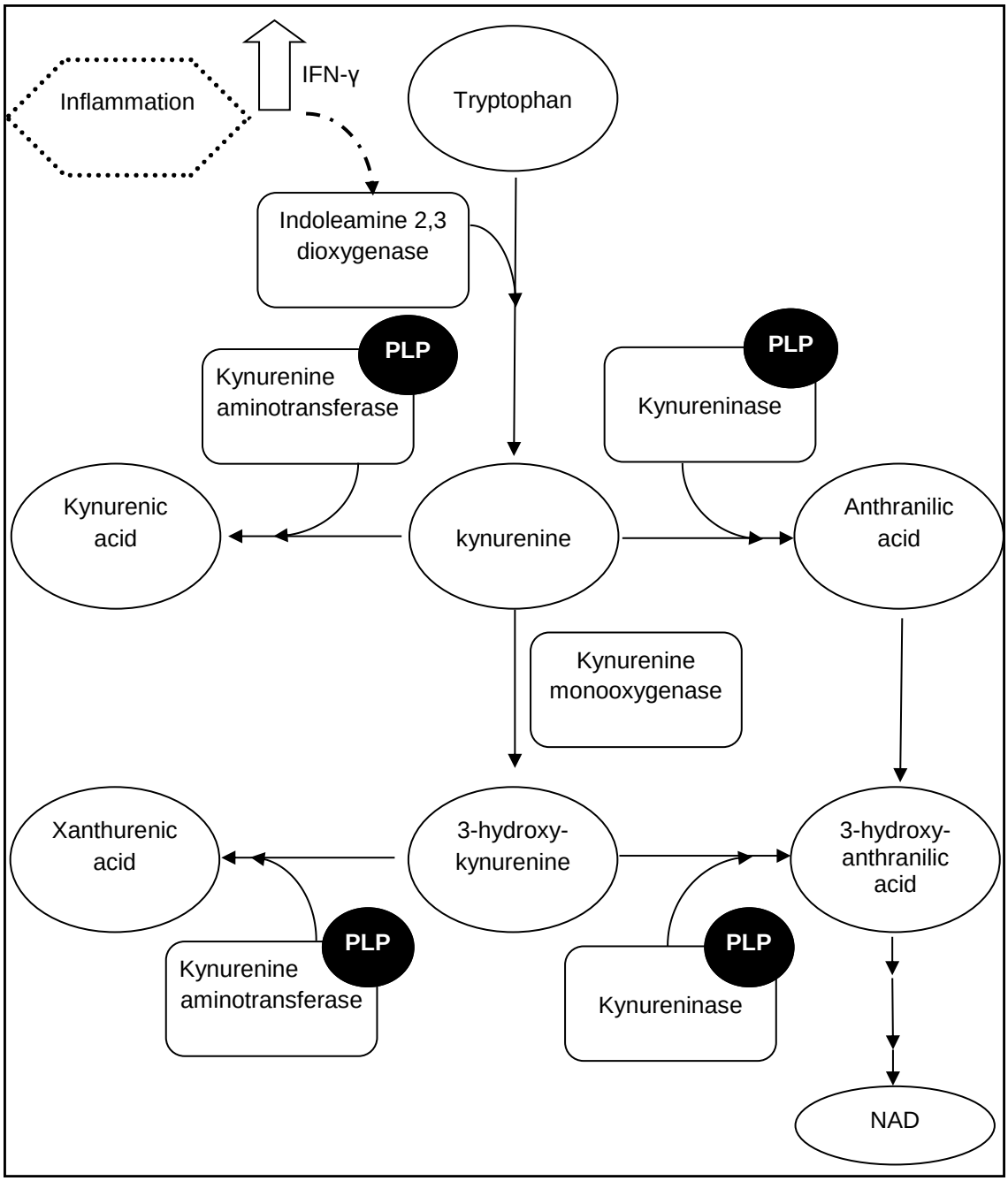

Figure 1: Illustration of the Kynurenine Pathway. During inflammation, macrophages and dendritic cells secrete interferon gamma (IFN-γ) which in turn increases expression of indoleamine 2,3 dioxygenase (IDO). Enzymes downstream of IDO require PLP to produce kynurenine metabolites.

Table 1. General characteristics of the Nutrition, Aging, and Memory in Elders cohort

	(n=915)	
Characteristic	mean	(SD)
Age (yrs)	75.29	(8.41)
BMI (kg/m^2)	31.60	(8.50)
Pyridoxal Phosphate (nmol/L)	68.32	(72.85)
Plasma folate (ng/mL)	14.90	(8.60)
Vitamin B-12 (pg/mL)	595.28	(534.39)
Homocysteine (μmol/L)	11.94	(5.23)
Creatinine	1.08	(0.97)
Vitamin B-6 intake (mg)	7.11	(19.10)
Tryptophan (μmol/L)	72.93	(37.99)
Kynurenine (μmol/L)	1.11	(0.72)
Anthranilic Acid	22.15	(17.66)
Kynurenic Acid	47.59	(27.94)
Hydroxykynurenine	54.55	(182.02)
Hydroxyanthranilic Acid	33.46	(26.39)
Xanthurenic Acid (nmol/L)	22.37	(27.75)
Neopterin	4.01	(9.46)

Characteristic	n (%)
Female	702 (76.72)
Current smoker	149 (16.28)
Diabetes	427 (36.56)
Low PLP (<30 nmol/L)	292 (31.91)
Race	
Non-Hispanic White	567 (61.97)
Non-Hispanic Black	328 (35.85)
Other race	20 (2.19)
Education	
Not High School grad	306 (33.44)
High School Grad	307 (33.55)
Some college/Bachelors	262 (28.63)
Grad School	40 (4.37)

NSAID – Nonsteroidal anti-inflammatory drug;

Table 2: Association of PLP with cognitive measures

Measure	Change in score per IQR	Standard Error	p-value
Factor Score for Memory	<-0.01	0.03	0.95
Factor Score for Attention	0.07	0.03	0.02
Factor Score for Executive Function	0.05	0.03	0.07
	Regression Coefficient	**Standard Error**	**p-value**
Mini-Mental State Examination*	0.22	0.10	0.03

Multiple Linear Regression analysis adjusted for plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, race, and diabetes status

*Tobit regression, upper bound censoring at 30, adjusted for plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status

Table 3: Association of kynurenine metabolites with Factor Score for Memory

Measure	Change in Memory per IQR	Standard Error	p-value
Tryptophan	0.03	0.04	0.49
Kynurenine	0.02	0.04	0.46
Anthranilic Acid	0.07	0.04	0.09
Kynurenic Acid	-0.09	0.05	0.09
Hydroxykynurenine	<0.01	<0.01	0.61
Hydroxyanthranilic Acid	-0.01	0.02	0.74
Xanthurenic Acid	-0.04	0.03	0.16
Kynurenine:Tryptophan Ratio	1.75	2.88	0.55

Multiple Linear Regression analysis adjusted for plasma PLP, plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status

Table 4: Association of kynurenine metabolites with Factor Score for Attention

Measure	Change in Attention per IQR	Standard Error	p-value
Tryptophan	0.02	0.04	0.64
Kynurenine	<-0.01	0.04	0.90
Anthranilic Acid	<-0.01	0.04	0.99
Kynurenic Acid	-0.02	0.05	0.74
Hydroxykynurenine	-0.01	0.01	0.18
Hydroxyanthranilic Acid	0.03	0.02	0.27
Xanthurenic Acid	0.02	0.03	0.51
Kynurenine:Tryptophan Ratio	1.58	2.87	0.59

Multiple Linear Regression analysis adjusted for plasma PLP, plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status

Table 5: Association of kynurenine metabolites with Factor Score for Executive Function

Measure	Change in Executive Function per IQR	Standard Error	p-value
Tryptophan	-0.03	0.03	0.43
Kynurenine	-0.01	0.04	0.82
Anthranilic Acid	0.03	0.03	0.43
Kynurenic Acid	0.08	0.05	0.10
Hydroxykynurenine	-0.05	0.01	0.41
Hydroxyanthranilic Acid	-0.02	0.02	0.42
Xanthurenic Acid	0.02	0.03	0.47
Kynurenine:Tryptophan Ratio	-1.19	2.65	0.66

Multiple Linear Regression analysis adjusted for plasma PLP, plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status

Table 6: Association of kynurenine metabolites with Mini Mental State Examination

Measure	Change in MMSE score per IQR	Standard Error	p-value
Tryptophan	0.05	0.13	0.73
Kynurenine	0.22	0.12	0.09
Anthranilic Acid	-0.03	0.07	0.68
Kynurenic Acid	0.02	0.16	0.90
Hydroxykynurenine	-0.01	0.03	0.78
Hydroxyanthranilic Acid	-0.04	0.08	0.63
Xanthurenic Acid	-0.02	0.08	0.83
Kynurenine:Tryptophan Ratio	3.38*	0.97	0.74

Tobit Regression analysis adjusted for plasma PLP, plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status

Table 7. Association of Kynurenine Metabolites with Odds of Depression

Measure	OR	95%CI
PLP	1.001	(0.999-1.003)
Tryptophan	0.997	(0.992-1.003)
Kynurenine	0.946	(0.690-1.298)
Anthranilic Acid	0.990	(0.971-1.009)
Kynurenic Acid	1.003	(0.995-1.011)
Hydroxykynurenine	0.999	(0.998-1.001)
Hydroxyanthranilic Acid	0.996	(0.986-1.006)
Xanthurenic Acid	1.004	(0.995-1.012)
KTR	2.091	(<0.001 - >999)

Logistic Regression predicting CESD score above 16, adjusted for plasma PLP*, plasma folate, plasma vitamin B12, homocysteine, creatinine, age, gender, BMI, education, smoking status, alcohol drinking status, hypertension, ApoE4, Race, and diabetes status. *Except for the first measure, plasma PLP itself.

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Chapter 6

Effect of Heart Healthy and Atherosclerotic diets, with and without atorvastatin, on kynurenine metabolites and plasma pyridoxal-5'-phosphate in a porcine model of atherosclerosis

Abstract

Cardiovascular disease (CVD) is associated with low plasma levels of the bioactive form of vitamin B6, pyridoxal 5'-phosphate (PLP), as well as increased tryptophan degradation to kynurenine metabolites via the kynurenine pathway. Kynurenine metabolites have immunomodulatory properties, and require PLP for their production. We hypothesize that in inflammatory diseases such as CVD, increased kynurenine metabolism at sites of inflammation reduces the available plasma PLP stores. We tested this hypothesis by examining the relation between PLP, kynurenine metabolites, and markers of inflammation in aorta and plasma samples from a porcine model of diet induced atherosclerosis. Thirty-two Ossabaw pigs were randomized to one of four treatment groups: atherosclerotic diet, atherosclerotic diet plus atorvastatin, heart healthy diet, or heart healthy diet plus atorvastatin for 6 months. PLP and kynurenine metabolites were measured in aorta and plasma samples using LCMS/MS, inflammatory markers tumor necrosis factor α and high sensitivity C-reactive protein were measured in plasma using immunoassay. After 6 months of treatment, there were no significant differences between treatment groups with regards to plasma tumor necrosis factor α and high sensitivity C-reactive protein concentrations. Only plasma hydroxykynurenine differed between treatment groups: atherosclerotic diet alone resulted in plasma hydroxykynurenine concentrations 5.8 times higher than that of pigs on the same diet plus atorvastatin ($p=0.02$) and 2.9 times that of pigs on a heart healthy diet alone ($p=0.04$). Atorvastatin treatment resulted in higher plasma concentrations of aortic KA and XA concentrations and plasma neopterin concentrations than that of pigs on diet alone. No significant differences were observed in other kynurenine metabolites or PLP

between treatment groups in plasma or aortic tissues. These observations were ultimately observed in the absence of any difference in inflammation status, and are thus difficult to interpret with respect to the effect of atherosclerotic inflammation on kynurenine metabolism and distribution of PLP at sites of inflammation. Larger studies with longer treatment duration may be required to generate a greater immune response and definitively determine the relation between PLP and kynurenine at sites of inflammation.

Background

Cardiovascular Disease (CVD) is one of the leading causes of death in the United States [1]. We now understand CVD to be a disease of inflammation, with increased immune activity at sites of atherosclerosis. CVD is associated with decreased plasma concentrations of the bioactive form of vitamin B6, pyridoxal-5'-phosphate [2]. We continue to observe low plasma PLP status associated with higher levels of inflammation in the general population as well, across all levels of vitamin B6 intake [3].

Inflammation is associated with increased degradation of tryptophan via the kynurenine pathway by induction of the enzyme indoleamine 2, 3 dioxygenase (IDO). IDO is present in immune tissues and is induced by inflammatory cytokines. Certain PLP-dependent enzymes downstream of IDO require PLP as a cofactor (Figure 1). Kynurenine aminotransferase requires PLP to convert kynurenine to kynurenic acid (KA) and 3-hydoxykynurenine (HK) to xanthurenic acid (XA). Kynureninase requires PLP to convert kynurenine to anthranilic acid (AA) and HK to 3-hydroxyanthranilic acid (HAA). Physiologically, kynurenine metabolites can induce a variety of changes, which may alter the risk of developing CVD. For example, kynurenine has been demonstrated to be a

vasodilator, and may play a role in reducing blood pressure, an important component of cardiovascular health [4].

Certain kynurenine metabolites and IDO activation may play a key role in atherosclerosis. A study by Cole et al. found that in hypercholesterolemic APOe$^{-/-}$ mice IDO$^{-/-}$ knockout (IDO-)has more aortic lesions, greater plaque area percentage, and more indicators of plaque instability than IDO$^{+/+}$ (IDO^{+}) mice [5]. Plasma analysis of the IDO- mice showed decreased kynurenine concentrations, but no difference in tryptophan when compared to IDO^{+} mice. [5 Other cell culture studies demonstrated 3-hydroxyanthranilic acid can induce T-cell apoptosis and has been shown to reduce recruitment of leukocytes to endothelial cells via reduction of both monocyte chemoattractant protein-1 secretion and vascular cell adhesion molecule-1expression {Hayashi, 2007 #104, 6]. Kynurenine pathway activation may also reduce inflammatory cell migration, an important component in the development of atherosclerotic plaques, as suggested by a study wherein the kynurenine pathway was inactivated in IDO knockout mice. IDO-/- mice had increased inflammatory cell infiltration in liver during diet induced steatohepatitis [7].These results suggest certain kynurenine metabolites, rather than depletion of tryptophan, are active in the atherosclerotic process [5].

Increased plasma kynurenine to tryptophan ratio, a marker of kynurenine pathway activation, is seen in subjects with cardiovascular disease and has been shown to be indicative of inflammation severity [8, 9]. The kynurenine to tryptophan ratio is also a prognostic indicator for mortality post ischemia [10] and a good predictor of coronary

events in subjects without CVD [11]. Such findings indicate the kynurenine pathway may play an important role in CVD development.

To treat or prevent CVD, the CDC reports that approximately 27% of adults in the United States aged 40 and over, and 40% of adults aged 65 and older, take a cholesterol lowering medication, such as a statin. [12]. Statins have been demonstrated to reduce general markers of inflammation, such as C-reactive protein, inhibit release of IFN-γ, and limit T-cell proliferation through inhibition of the IFN-γ induced expression of major histocompatibility complex-II in human endothelial cells and macrophages [13-15]. It is through statin's effects on IFN-γ that we suggest it may lower kynurenine pathway activation and PLP mobilization.

No studies thus far have investigated PLP or kynurenine pathway activation at the site of inflammation. In the present study we examined the effects of atherosclerotic diet, heart healthy diet, and atorvastatin on concentrations of PLP, kynurenine metabolites, and neopterin, a measure of IFN-γ activation, in plasma, aortic tissue, and lymph nodes. Our hypothesis is that during atherosclerosis, PLP is mobilized from circulation to inflamed vascular tissue for use in the kynurenine pathway to regulate the immune response. We hypothesized that statins may reduce kynurenine pathway activation and normalize PLP levels through its anti-inflammatory effects.

Methods

Research animals:

12-week old male wild type Ossabaw pigs were used as an animal model for primary prevention of atherosclerosis through dietary and pharmacological means. Ossabaw pigs were selected for their small body size, slow growth rate, and ability to develop metabolic syndrome and subsequent atherosclerosis in response to dietary intervention alone [16]. All research activities handling live-animals were conducted at Beltsville Human Nutrition Research Center, at Beltsville Agricultural Research Center (USDA BNRC). Two pigs were housed per pen; each pen was equipped with heat lamps for comfort and a toy for mental stimulation. All animal handling procedures have been approved by the Beltsville Animal Care and Use Committee (BAACUC) to ensure proper handling and care to minimize pain and distress to animals during treatment and euthanasia (Protocol # 12-021). Pigs were euthanized humanely by an overdose of 26% sodium pentobarbital solution. This method is approved by the Department of Veterinary Medical Association Guidelines on Euthanasia.

Study design:

This was an ancillary study to the parent study aimed to assess the primary prevention of atherosclerosis through statin use in Ossabaw pigs. Pigs have similar cardiovascular anatomy to that of humans, and in particular, the Ossabaw pig is the only breed of pig known to develop metabolic syndrome and subsequently atherosclerosis as a result of dietary intervention alone [16]. Thirty-two pigs were randomized to one of four treatment groups (8 pigs per group) utilizing a 2x2 factorial design to assess the diet-drug

interaction in a pig model of diet-induced atherosclerosis. Pigs were randomized to either: 1) a heart healthy diet, 2) heart healthy diet plus atorvastatin, 3) atherogenic diet, or 4) atherogenic diet with atorvastatin. Pigs were treated for 6 months. The heart healthy diet was composed of 35% of total calories from fat, consisting of 7% saturated fat, 15% monounsaturated fat, and 13% polyunsaturated fat. Atherogenic diet was composed of 35% total calories from fat, consisting of 20% saturated fat, 10% monounsaturated fat, and 5% polyunsaturated fat. Weighed diet was administered to pigs as pre-weighed amounts, determined as a function of their body size to maintain growth. Water was provided ad libitum. Statin was administered in a daily dose similar to therapeutic dose for humans: approximately 1 mg/kg body weight.

At the end of the study, twenty-eight pigs were left for analysis (Table 1). Blood samples were collected in tubes coated with K_2EDTA and centrifuged to separate plasma from blood cells. Two samples of aortic tissues were taken from each pig: one from a plaque-prone area, at the beginning of the descending aorta, and one from a plaque-less area (an area less prone to plaque formation) from an area approximately 4 cm down the descending aorta. Samples of lymph nodes were also collected. Plasma and aortic samples were flash frozen in liquid nitrogen and shipped frozen to JM USDA HNRCA. Upon arrival of samples at JM USDA HNRCA, samples were stored at -70°C until analysis.

Measurement of kynurenines:

Plasma tryptophan metabolites (kynurenic acid, kynurenine, anthranilic acid, 3-hydoxykynurenine 3-hydroxyanthrnailic acid, anthranilic acid, tryptophan, xanthurenic acid) were measured simultaneously using the methods described by Midttun et al. [17]. Briefly, weighed and frozen aorta samples were homogenized in cold water. Sixty microliters of the aorta homogenate, thawed plasma, or red blood cell samples were deprotonated using a 1:1 ratio of plasma to 6% (w/v) trichloroacetic acid that was spiked with 100 nmol/mL deuterated kynurenine metabolite standards (US Biological, Salem, MA). The mixture was immediately vortexed, and shaken at 4°C for 1 hour. Samples were then centrifuged at 13,000 rpm for 30 minutes. The supernatant was filtered through 0.22 μm Durapore® filter plates (Milipore, Cork, IRL) and sample was loaded into glass vial inserts for LC/MS-MS analysis. An Agilent 1300 series HPLC system was equipped an-line filter (2 mm, 0.2 μm, 70x 0.12mm) and a Zorbax Eclipse XDB-C8 Rapid Resolution HT reversed-phase C-8 column (2.1 x 50 mm, 1. 8 μm) with Poroshell 120EC-C8 2.1 mm ultra-high pressure liquid chromatography (UHPLC) guard column in a thermostated column compartment set to 40°C. The HPLC was used with an AB Sciex 5500 linear ion trap tandem mass spectrometer with TurboSpray ion source. The mobile phase solutions were A: 650 mM acetic acid, B: 100 mM heptafluorobutyric acid and C: 90% (v/v) acetonitrile in water. A thermostated autosampler compartment kept samples at 4°C. Injection volume was 5 μL. Mobile phase solutions flowed constantly at a rate of 0.4 mL/minute in the following ratios at the designated time points: 0 minutes: 98% A, 2% B; 2.2 minutes: 78% A, 2% B, 20% C; 2.3 minutes: 60% A, 2% B, 38% C; 3.3 minutes: 40% A, 2% B, 58% C; 3.9 minutes: 2% A, 2% B, 96% C; 4 minutes: 98% A, 2% B; 6

minutes 98%A, 2%B. The total run time was 6.5 minutes. The mass spectrometer was set to positive polarity in multiple reaction monitoring mode, using an ion source temperature of 550°C and voltage of 5500 V.

Measurement of Inflammatory cytokines:

Inflammatory markers were measured by the Cardiovascular Nutrition Laboratory. Plasma concentrations of high sensitivity C-reactive protein (hsCRP) were measured using two-site enzyme linked immunoassay (Pig High-Sensitive CRP ELISA Cat. No. KT-184, Kamiya Biomedical Company, Seattle, WA 98168). Tumor necrosis factor alpha (TNFα) was measured by solid phase enzyme-linked immunoassay, **(**TNF-α Immunoassay Quantikine® ELISA kit, R&D Systems, Minneapolis, MN).

Statistical analysis:

We conducted separate analyses examining the effect of atherogenic diet on PLP and PA concentrations in plasma, and endothelial, intima-medial and adventitial aortic pieces. Two-factor ANOVA with diet as one factor and statin use as the second was used to assess the presence of a statistical interaction between diet and statin on kynurenine metabolite concentrations. If the cross-product term of diet×statin was not a statistically significant predictor of kynurenine metabolite concentrations ($p<0.05$), the main effects of statin and diet alone were assessed using Student's T-test at the 0.05 level of significance.

Results

In plasma samples, the interaction between diet and statin was statistically significant when predicting hydroxykynurenine (p=0.03), but only after removal of one outlier in the heart-healthy diet only group. The outlier was found to be greater than 3 standard deviations from the average HK of all pigs. When this observation was retained, there were no significant effects of diet, statin, or the interaction between the two (data not shown). Pigs on atherosclerotic diet alone had the greatest concentration of plasma hydroxykynurenine. On average, pigs on atherosclerotic diet had hydroxykynurenine concentrations 5.8 times that of pigs on the same diet plus atorvastatin (p=0.02) and 2.9 times that of pigs on a heart healthy diet only (p=0.04) (Figure 1). There were no other statistically significant interactions observed for any other kynurenine metabolite, PLP, or neopterin in plasma.

Pigs given atorvastatin had greater kynurenic acid concentrations (p=0.01) after the removal of one observation from the diet only group (Figure 2A). The outlier was more than 3 standard deviations from the mean KA concentration. When the outlier was included in the analysis, there was no significant difference between pigs treated with and without statin (p=0.60, data not shown). Pigs given atorvastatin also had greater xanthurenic acid concentrations than pigs on diet only, after removal of one outlier. The outlier was found to be greater than 3 standard deviations away from the average XA of all pigs. When this observation was retained, there were no significant effects of statin (p=0.46, data not shown). On average, aorta from plaque-less regions from pigs on atorvastatin had 1.3 nmol/g protein more XA than pigs not on atorvastatin, (95%CI: 0.56-

2.10) and since the variability among pigs on atorvastatin was much greater than that of pigs on diet alone, we used a Satterthwaite approximation for standard errors when conducting the t-test (p=0.002). Pigs on atorvastatin also had higher plasma neopterin than pigs receiving no statin (difference: 2.1 nmol/L (95%CI:2.1-4.03) p=0.03) (Figure 2C).

In homogenized aortic samples from plaque-less areas, pigs on heart healthy diet had higher hydroxyanthranilic acid than pigs on atherosclerotic diet (p=0.01) (Figure 3).

There was no difference in plasma concentrations of inflammatory biomarkers hsCRP and TNFα between diets or statin treatments. No significant effects of diet or statin were observed in kynurenine metabolite concentrations in aortic samples from plaque-prone area, or lymph nodes.

Discussion

In this project we found sporadic effects of diet and statin use on kynurenine metabolites. We also found heart healthy diet increased HAA concentrations and statin use increased KA, XA, and neopterin concentrations in plaque-less aortic samples. Both kynurenic acid and xanthurenic acid are products of the enzyme kynurenine aminotransferase. A previous study in stroke patients found lowered kynurenine aminotransferase activity and increased IDO activity in patients with stroke compared to controls [18]. We did observe that statin use was associated with increased products of KAT activity KA and XA within aortic tissues, which supports the previous findings.

However, against expectation, we did not observe an anti-inflammatory effect of statin since statin did not impact hsCRP or aortic neopterin concentrations, a measure of IFN-γ activation. Therefore, the effect of statin on inflammation and the kynurenine pathway remains inconclusive. Importantly, there was a lack of effect of diet and statin on inflammation. This could be due to inadequate treatment duration and high variability in response due to use of wild-type animals. Ultimately, this means that we could only observe effect of diet and statin on kynurenines when inflammation remains unchanged, which would explain the conspicuous lack of associations observed.

Thus far, the information linking the kynurenine pathway to cardiovascular disease is mostly from epidemiological studies which examined kynurenine pathway activation by measuring circulating biomarkers such as plasma kynurenine to tryptophan ratio [9-11, 19, 20]. Several studies have investigated the relationship between inflammatory cytokines and kynurenines in vitro; however, these studies cannot take into account the complexity of the immune response in different organs during diet-induced atherosclerosis. The use of pigs as an animal model allowed for analysis of tissues otherwise unattainable from human subjects. This study was designed as an ancillary study to a previously approved project with the goal that animal studies should make the most complete use of all tissues available from animal studies in order to minimize the number of animals used for scientific research. However, the use of outbred Ossabaw pigs, and the small sample size of 8 pigs per group may have diminished our opportunity to detect differences in kynurenine metabolite concentrations. Wild-type animals vary in response to diet and statin, which contributed to the large variability in our data, and this

may represent a key factor limiting our ability to detect the differences even if they indeed exist.

This project investigated kynurenine pathway metabolites in the context of atherosclerosis using an animal model that is ideally suited to mimic the pathogenesis of atherosclerosis in humans. We were able to observe the effects of diet or statin on kynurenine metabolites, in the absence of changes in inflammation. Future studies that successfully create a diet or statin induced difference in inflammation are necessary to fully discern the role of PLP and kynurenine pathway activation at sites of inflammation.

Figures and Tables

Table 1. Final number of pigs per treatment group

	Atherogenic Diet	Heart Healthy Diet
No Statin	7	6
Atorvastatin	7	8

Figure 1

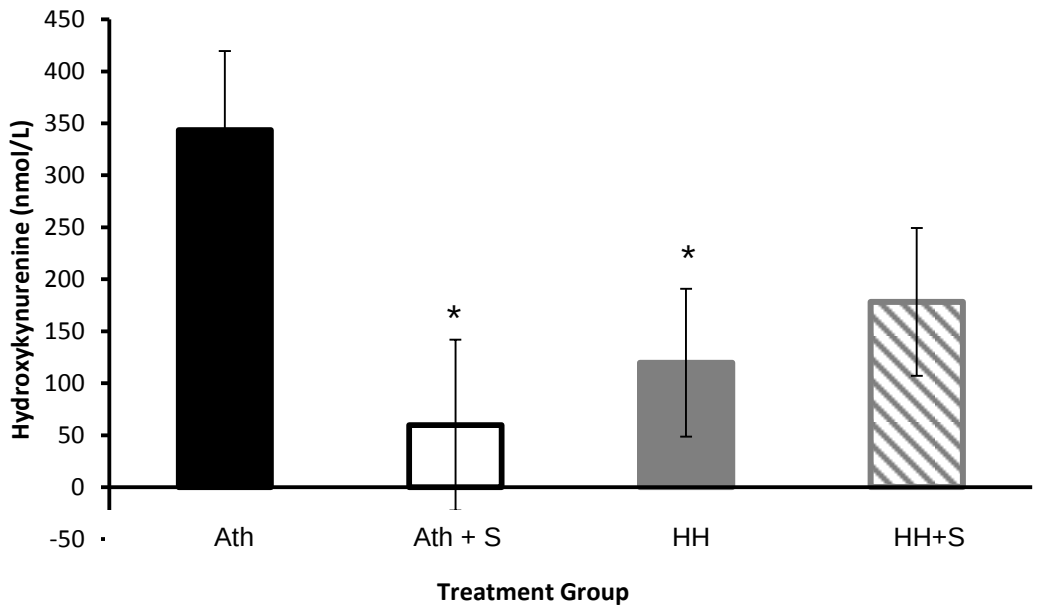
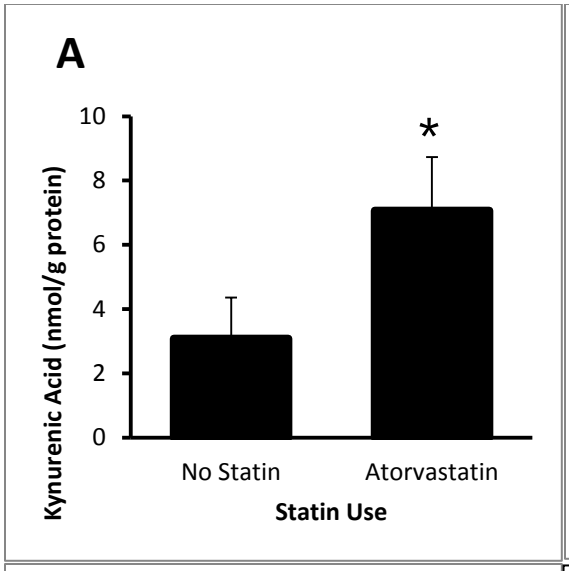
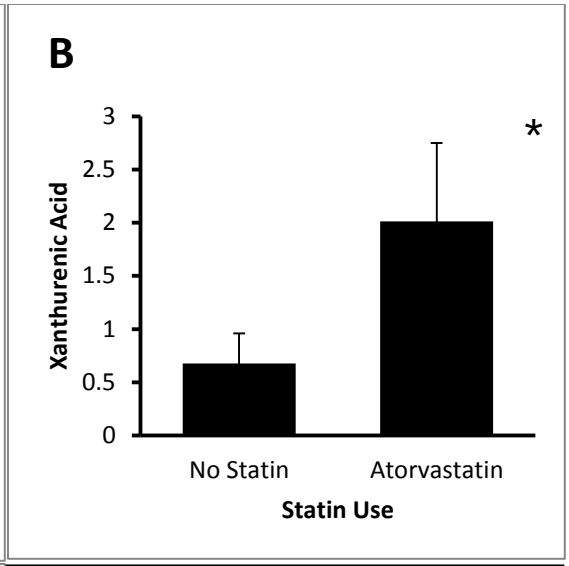


Figure 1: Plasma Hydroxykynurenine by Treatment Group
* indicates significantly different from Ath group ($p<0.05$).
Ath – Atherosclerotic Diet, Ath +S - Atherosclerotic Diet + Atorvastatin, HH – Heart Healthy Diet, HH+S – Heart Healthy Diet + Atorvastatin





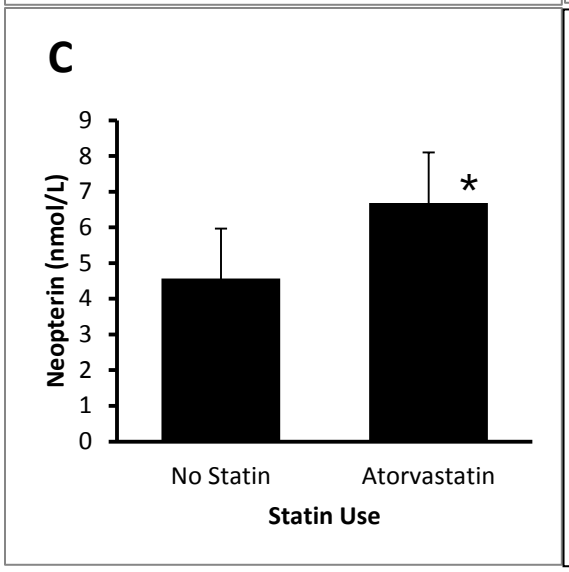


Figure 2: Effect of statin on kynurenine metabolites in various tissues. * indicates difference from No Statin is statistically significant ($p<0.05$). Bars represent 95% CI.

A: Kynurenic acid concentration in aortic samples from plaque-less area; B: Xanthurenic acid concentration from aortic samples in plaque-less area; C: Neopterin concentration in plasma samples

Figure 3

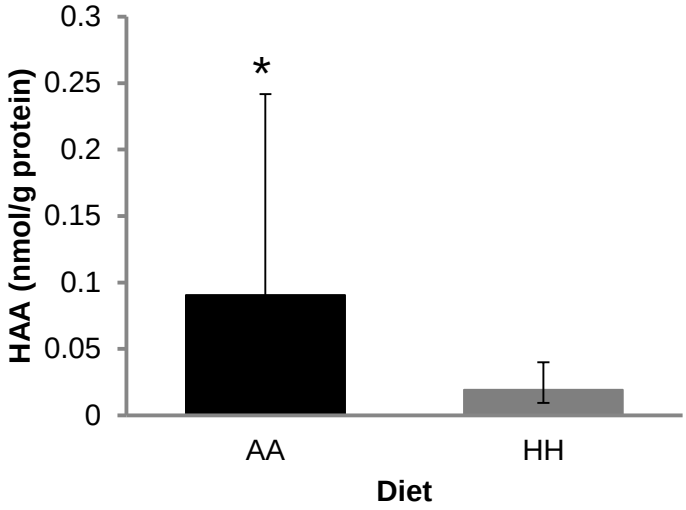


Figure 3: Effect of diet on hydroxyanthranilic acid in plaque-less aortic tissue. * indicates difference from heart healthy diet is statistically significant ($p<0.05$). Bars represent 95% CI.

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Chapter 7

Conclusions and Future Directions

Limitations

There are several methodological aspects to the work presented here that limit the results of our findings. The analysis from Chapter 2 used kynurenine metabolite data that was generated as part of a larger metabolomics profile. Only 3 out of 6 kynurenine metabolites were measured, limiting our ability to examine relations between PLP and all the kynurenine metabolites that are products of PLP-dependent reactions. This was addressed by performing a targeted metabolite profile on all the kynurenine metabolites in plasma from NAME. Both analysis of data from the Framingham Offspring Cohort as well as analysis of samples and data from the NAME were conducted cross-sectionally, precluding our ability to make any conclusions on causality or temporal relation between PLP and inflammation. While the results of these studies are able to provide us with evidence of correlation, longitudinal studies are required to determine a more causal relation between PLP and inflammatory mechanisms. The work conducted in Chapters 2 through 5 were limited to previously collected data and plasma samples available, and which did provide any insightful data about what might be happening at sites of inflammation such as organs or tissues. The work in Chapter 6 was meant to explore sites of inflammation during diet induced atherosclerosis, however there were no significant differences in inflammatory markers between treatment groups. This could have been due to the use of a small number of outbred wild-type Ossabaw pigs, which was likely the reason for such high variability in response to diet and statin treatments. Additionally, 6 months of treatment may not have been long enough to elicit an inflammatory response severe enough for our detection. While our study of porcine atherosclerosis was unable to generate an adequate inflammatory response, future studies in this field should use longer

treatment duration, with larger sample sizes per treatment group, to ensure severe inflammation occurs.

Summary of Novel Findings

The work presented here was designed to examine the relation between vitamin B6 and inflammation through the role of PLP as a cofactor in tryptophan degradation via the kynurenine pathway. Observations of low circulating PLP levels in subjects with inflammation and inflammatory conditions have been well established and were summarized in the literature review in **Chapter 1**. There have been no conclusive findings on the reason for low circulating PLP during inflammation. The present body of work sought to determine if lower circulating PLP levels are due in part to the utilization of PLP as a cofactor for reactions in the kynurenine pathway. Kynurenine pathway metabolites are immunomodulatory, and their increased production in inflamed tissues to reduce cell damage due to inflammation could explain the decreased availability of PLP in circulation.

In **Chapter 2** we tested this hypothesis in the context of prevalent CVD using data from the Framingham Heart Study, Offspring Cohort at Exam 5. As part of a metabolite profiling, tryptophan and 3 kynurenine metabolites (AA, KA, and HA) were previously measured plasma samples from this cohort. None of these metabolites were associated with CVD status. PLP insufficiency (<30 nmol/L) was more prevalent in subjects with CVD than in those without, and our results did not support the hypothesis that the association between plasma PLP concentrations and CVD status were due kynurenine pathway activation.

We then examined the same association in the context of type 2 diabetes with an ancillary study within the Nutrition, Aging, and Memory in Elders Study. In **Chapter 3** we measured kynurenine metabolites in plasma samples, and combined this data with previously collected data to determine if the association between plasma PLP and diabetes was due to kynurenine pathway activation. We found higher plasma AA, KA, and XA concentrations in diabetics compared to non-diabetics, and diabetics had lower plasma PLP than non-diabetics. When examining non-diabetic subjects we found KA was directly associated with HOMA2-IR score, a measure of insulin resistance. Furthermore, HOMA2-IR and plasma PLP were most strongly related amongst subjects with PLP insufficiency, and subjects with both high plasma XA and PLP insufficiency had the highest HOMA2-IR scores. We conclude that increased PLP and increased kynurenine metabolite production are associated with insulin resistance and prevalence of diabetes, however we did not find evidence to suggest increased kynurenine metabolite production underlies lowered plasma PLP concentrations in diabetes.

While there have been many observations of low plasma PLP in inflamed subjects, little research had been done to determine if there are adverse consequences of low plasma PLP. Using data from our analysis of plasma samples and previously collected data from NAME we examined if low plasma PLP would affect PLP-dependent reactions. PLP is required in both the synthesis of heme and the synthesis of neurotransmitters.

Chapter 4 examined if low plasma PLP concentrations observed during inflammation were also related to prevalence of anemia - which may arise from inadequate heme synthesis. Plasma PLP concentrations were inversely related to anemia prevalence, with

the highest prevalence of anemia observed in subjects with the lowest plasma PLP concentrations. Subjects in the lowest tertile category of plasma PLP with high plasma CRP concentration (>10 mg/L) had the highest prevalence of anemia compared to all other plasma CRP and PLP concentrations. These results supported the hypothesis that low plasma PLP concentrations observed during inflammation contribute to increased risk of adverse health outcomes related to PLP-dependent functions.

In **Chapter** 5 we used data from NAME and their cognitive measures to examine the relation between plasma PLP and measures of cognition and depression, which may arise from aberrant neurotransmitter synthesis. We observed that plasma PLP was directly related to factor scores for the attention domain of cognition, but not with any other domain, nor with cognitive impairment as assessed by MMSE, or depression as assessed by CES-D. None of the associations between cognition and kynurenine metabolites met statistical significance. These results suggest increased risk of cognitive impairment associated with inflammatory diseases may be explained by lower plasma PLP concentrations during inflammation.

Lastly, in **Chapter 6** we measured PLP and kynurenine metabolites in the aorta, lymph nodes, and plasma of pigs on either a heart healthy or an atherosclerotic diet, with or without atorvastatin. Data from analysis of inflammatory cytokines in plasma indicated no significant differences in inflammation between treatment groups, precluding our ability to observe differences in PLP or kynurenine metabolites due to inflammation. Atorvastatin treated pigs were observed to have greater XA and KA in aortic tissues and greater plasma neopterin concentration. Plasma HK was lowest in pigs on atherosclerotic diet treated with statin, on heart healthy diet without statin, when compared to pigs on

atherosclerotic diet alone. We were unable to come to any definitive conclusion regarding the distribution of PLP or kynurenine metabolites during atherosclerotic inflammation.

Future Directions

The work presented here does not provide rationale for future studies investigating increased kynurenine metabolism as an underlying cause of lower plasma PLP observed during inflammation. Future studies should explore the relations between inflammation and PLP or kynurenine pathway activation separately. Alternative mechanisms by which plasma PLP may be lowered include increased breakdown of PLP into metabolites such as pyridoxal and pyridoxic acid (PA) [1, 2]. While Chiang et al. have found no increase in urinary PA excretion in rheumatoid arthritis patients, Ulvik et al. observed increased PA:PLP ratio in plasma suggesting there is increased PLP catabolism during inflammation, even if PA is not ultimately excreted through urine. Only through additional animal studies on tissue distribution of PLP and PLP metabolites at sites of inflammation and excretion of PLP breakdown products will we gain a clear understanding of PLP catabolism during inflammation, and if it is ultimately the reason for lower plasma PLP observed in inflammatory conditions. Our work does support the hypothesis that insufficient PLP associated with inflammation may contribute to increased insulin resistance and ultimately diabetes. Longitudinal studies will be required to determine if decreases in plasma PLP precede increases in insulin resistance to ultimately determine temporality of this association.

References

1. Chiang, E.P., et al., *Plasma pyridoxal 5'-phosphate concentration is correlated with functional vitamin B-6 indices in patients with rheumatoid arthritis and marginal vitamin B-6 status.* J Nutr, 2003. **133**(4): p. 1056-9.

2. Ulvik, A., et al., *Evidence for increased catabolism of vitamin B-6 during systemic inflammation.* Am J Clin Nutr, 2014. **100**(1): p. 250-255.