Assessment and clinical implications of functional vitamin B6 deficiency
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1 Introduction
A Historical Perspective on the Discovery of Vitamins

Vitamins are essential to life and for prevention of diseases. It is difficult to imagine that, despite of their essentiality, they were only discovered somewhat more than a century ago. It were the observations of the Dutchmen Eijkman, Vorderman, and Grijns in the 1890s that gave birth to scientific research towards vitamins (1). They discovered that chickens only developed a characteristic leg weakness (polyneuritis) when they were being fed cooked white rice and that adding the “polishings” - i.e. the skin of brown rice - to chickens’ diet restored their health. Furthermore, the Dutchmen observed that in prisons using mostly brown rice, less than one prisoner in 10,000 suffered from the polyneuric disease beriberi, while in prisons using mainly white rice, this proportion was 1 in 39. When the observations of Eijkman, Vorderman, and Grijns became widely known, people started preparing active extracts from rice polishings that could be used to treat victims of beriberi, thereby demonstrating the solubility of these factors in water and alcohol.

Scientists around the world began to gain interest in the isolation of the factors from rice polishing, and began to think about how to identify and even synthesize them. One of these scientists was Casimir Funk, a biochemist born in Poland, who reported in 1911 that he had isolated the active factor, later known as thiamine or vitamin B1 (2), and suggested that this factor was part of the chemical class of “amines”. He further suggested that deficiency of these organic trace nutrients was also the cause of the fatal diseases pellagra and scurvy. Therefore, he collectively referred to these factors as “vital amines”. When scientists later realized that other factors were in fact no amines, the term was shortened to “vitamin”. After the initial discovery of thiamine, many other vitamins followed.

Vitamin supplementation has been shown to prevent diseases, such as neural tube defects and megaloblastic anemia in the case of folic acid (3) and rachitis and osteoporosis in the case of vitamin D (4). Over the past decades, vitamin research has evolved from studying overt vitamin deficiencies to more subtle, subclinical, deficiencies – i.e. insufficiencies. There has been increasing interest in whether correction of subclinical deficiencies, could result in the prevention or even the cure of age-related diseases like osteoporosis, dementia, diabetes, and cardiovascular disease (5, 6), and less clearly defined diseases and adverse health complaints, such as chronic fatigue syndrome and fibromyalgia (7, 8). Modern guidelines recommend vitamin supplementation only in subpopulations that are at risk of developing
overt vitamin deficiency, such as the pregnant, the elderly, and children (9). These recommendations are aimed towards prevention of deficiency-related conditions on the one hand, and prevention or amelioration of age-related diseases and health complaints on the other. However, the guidelines on supplementation are often general in nature and infrequently discriminate between different high-risk populations, while it is questionable whether a “one size fits all” approach is prudent and safe. Arguments against such an approach include the fact that diets vary considerably among individuals and that it is unclear whether regulatory authorities have taken this variation into account when designing the guidelines. On that note, it is important to realize that many foods are fortified with vitamins and that some of these foods might be significant contributors to vitamin intake (10).

There is increasing awareness that vitamin supplementation may not always be beneficial, and may even be harmful, by virtue of causing vitamin excesses. Accordingly, several randomized clinical trials have revealed that vitamin supplements may in fact increase risk of developing various conditions, such as cardiovascular disease (11), cancer (12), and overall mortality (13). These adverse effects, however, are not specific for research settings (14). A recent case example of overt toxicity associated with high-dose vitamin supplementation, was the case of Sven Kramer, a professional Dutch top distance speed skater. In compliance with recommendations made by the team’s physician, his diet consisted of high-dose supplementation of various vitamins, including vitamin B6. After complaints fitting with neuropathy, physicians found toxic levels of vitamin B6 in his circulation. Accordingly, the multivitamin supplements were removed from his diet, vitamin B6 levels soon returned to normal, and the complaints subsided. Strikingly, Kramer’s diet was prescribed even though it was unknown whether he was vitamin B6 deficient. This case exemplifies the lack of knowledge on the indications for and consequences of vitamin B6 supplementation, and indicates that vitamin B6 intake is clearly not a suitable reflection of one’s vitamin B6 requirement. However, vitamin intake does affect vitamin requirement. In addition, there are several other processes that could conceivably affect vitamin requirement, such as intestinal absorption, metabolism, and excretion (15). Furthermore, numerous lifestyle factors and physiological factors can have profound effects on the handling and thus circulating levels of a vitamin, albeit this change may not necessarily correspond to a change in intracellular availability of that vitamin (16). Lifestyle and physiological factors thereby have the potential to bias interpretation of circulating concentrations of a vitamin (17). Thus,
there is need for biomarkers that reflect intracellular vitamin. In the case of vitamin B6, several, so-termed, functional biomarkers have been deemed suitable for reliable assessment of intracellular vitamin B6 availability (18). These functional biomarkers will likely provide the opportunity to better define optimal vitamin B6 status than allowed by assessment of vitamin B6 intake or measurement of plasma vitamin B6 concentrations. We hypothesize that in the future, such functional markers may allow for better guidance of supplementation advice and for reaching optimal vitamin status without reaching levels of toxicity.

**Handling and Physiology of Vitamin B6**

Vitamin B6 is a generic term that signifies six inter-convertible compounds, including pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorylated derivatives pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP), figure 1 (17). The forms of vitamin B6 that are present in foods include PN, which mainly originates from plant tissue, and PL, PM, and the three phosphate esters which mainly originate from animal-based products (19). Upon ingestion, the dephosphorylated vitamin B6 species are almost entirely absorbed in the jejunum through passive diffusion and are subsequently transported to the liver where they are largely metabolized to the most bioactive vitamin B6 form: PLP, figure 2 (20, 21). Since PLP is water-soluble, it is transported to the circulation bound to albumin, which protects it from hydrolysis (17, 19). In circulation, the albumin-PLP complex accounts for >95% of circulating PLP and thus functions as the PLP pool available for uptake in tissue (22, 23). However, the hydrophilic PLP does not readily cross cell membranes and therefore requires dephosphorylation to the less hydrophilic PL by the circulating tissue non-specific enzyme alkaline phosphatase (ALP) (17). Once in the intracellular compartment, PL is phosphorylated back to the biochemically most prominent vitamin B6 species PLP, marking the final step of the dynamic and complex path that vitamin B6 covers from food to active sites in the human body. Eventually, the vitamin B6 species are converted in the liver to their bio-inactive catabolite 4-pyridoxic acid (PA) and excreted in urine (17).

The importance of vitamin B6 has been known since the mid-20th century, when controlled animal studies showed that pyridoxine-deprivation caused development of atherosclerotic lesions in monkeys (24, 25). The biochemical
impact of the most active vitamin B6 isoform, PLP, is vast, constituting >160 biochemical reactions that affect metabolism of amino acids, lipids, neurotransmitters, and immunomodulating compounds, among other (17). While many of the biochemical functions of PLP remain unknown, studies have crystallized its involvement in some pathways, such as the one-carbon metabolism pathway, in which vitamin B6 supports the degradation of the established cardiovascular risk factor homocysteine (Hcy) (26), and the kynurenine pathway of tryptophan degradation (27), as discussed in more detail further ahead.

Figure 1. Vitamin B6 isoforms and their metabolism.
ALP, alkaline phosphatase; AOX, aldehyde oxidases; AT, aminotransferase; PDXK, pyridoxal kinase; PDXP, pyridoxal phosphatase; PNPO, pyridoxamine 5’-phosphate oxidase. Figure adapted from Ueland et al 2015 (17).
Figure 2. Simplified schematic overview of vitamin B6 handling in humans.

Dashed lines indicate (diffuse) transport across biological membranes. Left right arrows indicate (bio)chemical equilibrium under steady state conditions. *General cellular compartment, including liver cells. 3-HK, 3-hydroxykynurenine; ALB, albumin; AS, active sites; PA, 4’-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5’-phosphate; PM, pyridoxamine; PN, pyridoxine; TRP, tryptophan; XA, xanthurenic acid. Figure modified from Ueland et al 2015 (17).
Biomarkers of vitamin B6 status

Direct markers

The most commonly used indicator to assess vitamin B6 status, is PLP measured in (deproteinized) plasma or whole blood. Plasma and whole blood PLP are considered direct markers, as they directly provide information on the amount of circulating PLP in plasma and erythrocytes (17). While the correlation between erythrocyte and plasma PLP concentrations in healthy subjects is high (correlation coefficients >0.80) (28), this correlation may be considerably lower in critically ill patients (correlation coefficients <0.45) (29, 30). Studies have suggested that this discrepancy might be a consequence of the high susceptibility of plasma PLP, but not erythrocyte PLP, to several physiological and lifestyle factors that are not necessarily related to vitamin B6 status. For example, inflammation has been hypothesized to lower plasma PLP concentrations through several ways, including redistribution of PLP from plasma to muscles and increased catabolism of PLP to PA (possibly through low albumin levels) (31). Other important factors that could adversely affect plasma PLP concentration, include smoking, chronic alcohol use, pregnancy, certain drugs, low serum albumin and high alkaline phosphatase activity (17). Nonetheless, the within-person variance of plasma PLP concentration is considered relatively low (intra-class correlation coefficient (ICC) of approximately 0.65), allowing the use of plasma PLP for one-exposure assessment of vitamin B6 status under physiological conditions (32).

Erythrocyte PLP is considered a potentially more relevant indicator of vitamin B6 status than plasma PLP, especially in diseased populations, as it is thought to be less susceptible to potential confounders (17). However, the fact that PLP must be measured in freshly obtained erythrocytes (33), makes its use in large scale population studies less feasible, since samples are typically stored over a period of time before use for biochemical analysis. The resultant lack of large-scale population-based data on erythrocyte PLP, has hampered its use as marker of long-term vitamin B6 status in scientific studies and has, at least in part, contributed to plasma PLP as the most frequently used direct marker of vitamin B6 status in scientific studies.

Functional markers

Functional markers of vitamin B6 are thought to reflect the availability of PLP at active sites, i.e. intracellular enzymes that require PLP as co-factor (17).
Most functional markers estimate the intracellular activity of a PLP-dependent enzyme by reflecting the absolute or relative concentration of a substrate and/or product of such an enzyme in plasma or urine (17). Recent efforts towards developing and characterizing new functional markers of vitamin B6, have yielded promising functional markers, such as the ratio between two components of the kynurenine pathway of tryptophan degradation: 3-hydroxykynurenine (3-HK) and xanthurenic acid (XA), figure 2 (18). The 3-HK/XA ratio estimates the activity of the intracellular enzyme kynurenine aminotransferase (KAT), which requires PLP to maintain is biochemical function. Consequently, a low intracellular bioavailability of PLP will impair KAT activity and result in an increased 3-HK/XA ratio. Important advantages of this functional vitamin B6 marker include its responsiveness to vitamin B6 supplementation, and, notably, its weak association with inflammation (18). Moreover, as with plasma PLP, the within-person variance of 3-HK and XA over 1-3 years is relatively low, with ICCs ranging from 0.5 to 0.7 (32). Furthermore, population-based prospective studies have associated a higher 3-HK/XA ratio with increased risk of cancer events (34, 35), which suggests that this marker holds clinical relevance.

Kynurenine Pathway of Tryptophan Degradation
The vast majority of the essential amino acid tryptophan (Trp) is used for synthesis of proteins (36). However, a small proportion – approximately 1% – of ingested Trp will have a different metabolic fate, i.e. degradation via four different pathways (37). While three of these pathways, i.e. hydroxylation to serotonin and melatonin in the brain, decarboxylation to tryptamine, and transamination to indolepyruvic acid, are of minor quantitative significance, the fourth, so-called kynurenine pathway, is responsible for approximately 95% of tryptophan degradation under physiological conditions (38). The first and rate-limiting step of the kynurenine pathway is the conversion of tryptophan to formylkynurenine (FK) by the constitutively expressed liver enzyme tryptophan 2,3-dioxygenase (TDO) and the inducible tissue non-specific enzyme indoleamine 2,3-dioxygenase (IDO), figure 3 (39). IDO exists in two isoforms – IDO-1 and less known IDO-2 – but only the former is thought to play a meaningful role in the kynurenine pathway (40). After the initial conversion, FK is converted to kynurenine (Kyn), which is then further metabolized to either 3-HK by the flavine adenine dinucleotide (FAD)-dependent enzyme kynurenine 3-monooxygenase (KMO), or to kynurenic acid (KA) or anthranilic acid (AA) by the PLP-dependent enzymes...
KAT and kynureninase (KYNU), respectively (31). The same PLP-dependent enzymes subsequently convert 3-HK to either XA or 3-hydroxyanthranilic acid (HAA), respectively. Ultimately, HAA either yields carbon dioxide and water through formation of acetyl Co-A, or the final metabolite nicotinamide adenine dinucleotide (NAD\(^+\)). However, the main flow of metabolites through the kynurenine pathway is via Kyn, 3-HK, and HAA to produce NAD\(^+\) (41). The kynurenine pathway constituents are mainly excreted via urine, and thus their plasma concentration depends heavily on kidney function (39).

**Figure 3. Simplified schematic overview of the kynurenine pathway.**

Acetyl Co-A, acetyl coenzyme A; FAD, flavin adenine dinucleotide; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine transaminase; KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; NAD, nicotinamide adenine dinucleotide; PLP, pyridoxal 5’-phosphate. Figure modified from Ueland et al 2017 (31).

The kynurenine pathway comprises several enzymes and biologically active compounds that have been implicated in manifold diseases, particularly those associated with dysfunction of the immune response and of the central nervous system (42). Among the most investigated pathway constituents,
are Trp, Kyn, and 3-HK. While Tryp and Kyn are relatively inert compounds, compared to 3-HK, their ratio (Kyn/Trp) has been widely used to estimate IDO activity (43, 44). Increased IDO activity has been linked to inflammatory response and, specifically, is thought serve as an indicator of Th-1-type immune activation (45). IDO activity has therefore been appreciated as a potential modifiable factor in various immune-related conditions, such as allograft rejection and cancer (46, 47). One of the most bioactive constituents of the kynurenine pathway is 3-HK. While it has been shown that 3-HK can have a dual redox role by either serving as a pro- or as an antioxidant under specific conditions, it is generally perceived as an endogenous generator of reactive oxygen species (48, 49). From this point of view, increased 3-HK concentrations have been linked to neurological and cardiovascular disorders (50).

**Aims and Outline of this Thesis**

The overall aims of this thesis are to investigate the potential effects of vitamin supplementation in fibromyalgia (FMS) and chronic fatigue syndrome (CFS) and to evaluate and compare functional vitamin B6 markers with vitamin B6 status markers and vitamin B6 intake in various populations.

FMS and CFS are disabling disorders that are thought to share etiological pathways (51). Although the causes and underlying mechanisms of these disorders are unknown, it has been suggested that deficiencies of certain vitamins may be involved (7, 8). Today, the potential role of vitamins in FMS and CFS is subject of much debate, which is continuously fueled by a steady supply of conflicting data (52, 53). Notwithstanding, studies in favor of a pathophysiological role of vitamins and minerals have prompted physicians to recommend and patients to take micronutrient supplements, often in high doses, to alleviate symptoms (53-57). However, scientific evidence for such use of micronutrients is far from conclusive and these patients might unnecessarily put themselves at risk of vitamin and mineral toxicity. Therefore, in chapter 1, we assessed the available evidence for a role of vitamins and minerals in FMS and CFS in a systematic review and meta-analysis.

One of the vitamins that is present in virtually all plain multivitamin preparations, is vitamin B6. Early studies had postulated that vitamin B6 deficiency, like vitamin B12 and folate deficiency, was a risk factor for
cardiovascular disease due to the involvement of PLP in Hcy degradation (58, 59). Recent data, however, has suggested that the association between vitamin B6 and cardiovascular disease may not or not entirely be related to increased Hcy levels (60), but that it might be secondary to inflammation (61, 62). Indeed, the strong association between vitamin B6 and the inflammation parameter hs-CRP is independent of Hcy (63), and both parameters were found to be independently associated with coronary artery disease (64). However, the role of inflammation in the association between vitamin B6 and cardiovascular disease has not been formally investigated in the general population. In chapter 2, we hence assessed whether vitamin B6 deficiency is an independent risk factor for cardiovascular outcome in the general population, with specific consideration of the potential involvement of inflammation.

The general populations of developed countries have a low prevalence of vitamin B6 deficiency, due to the relatively high amounts of vitamin B6 present in foods (17). Nonetheless, several subpopulations are at increased risk for developing vitamin B6 deficiency, compared to the general population. For example, vitamin B6 deficiency, identified by plasma PLP concentrations below 20 nmol/L, has been frequently observed in renal transplant recipients (RTR) (65, 66). However, it is not known whether vitamin B6 deficiency may be caused by inadequate vitamin B6, nor whether vitamin B6 has long-term clinical implications in RTR. In addition, it is unclear whether low plasma PLP concentrations reflect a low functional vitamin B6 status, as assessed by the 3-HK/XA ratio, and whether a low functional vitamin B6 could have relevant clinical implications in this subpopulation. Therefore, in chapter 3, we measured plasma PLP in a large prospective cohort of stable RTR to investigate whether vitamin B6 deficient RTR might be at increased risk of adverse long-term outcome. In chapter 4, we subsequently studied vitamin B6 functionality in RTR and investigated the potential link between functional vitamin B6 status and long-term outcome in RTR. As touched upon in the introduction, many enzymes involved in the kynurenine pathway, like kynurenine transaminase, depend on PLP for proper function. Since RTR are at risk of developing vitamin B6 deficiency, the function of key enzymes of the kynurenine pathway might be disturbed in this population. Chronic low-grade inflammation, which is frequently present in RTR, might additionally affect these enzymes. Consequently, these disturbances might impair flow through the kynurenine pathway and give rise to an aberrant kynurenine profile, of which the potential implications in RTR are largely unknown (67-
69). In Chapter 5, we therefore investigated the potential long-term clinical implications of activation of the kynurenine pathway in RTR. To this end, we measured Trp, Kyn, and 3-HK in plasma and urine of a large cohort of stable RTR. Additionally, we estimated enzyme activities of IDO and KMO by calculating the Kyn/Trp and 3-HK/Kyn ratios.
References


