7 Summary, General Discussion, and Future Perspectives
Prelude

With improved diet quality, hygiene, and health care, general life expectancy has risen from 61.7 years in 1980 to 71.8 years in 2015, albeit the commonly known sex-specific difference has not markedly been affected (1). However, the gain in life expectancy is accompanied by an increased burden of non-communicable diseases that not only negatively affects quality of life, but also contributes to the upward trends of national health care expenses (2). Of approximately 57 million deaths globally in 2015, 40 million (71%) were attributable to non-communicable diseases (NCDs), which particularly comprised cardiovascular diseases (18 million, 45% of NCD total) and cancer (9 million, 22% of NCD total) (3). In search of modifiable factors to lower burden of existing NCDs and to prevent or delay manifestation of future NCDs, vitamins have gained considerable attention. Over the past decades, public interest in vitamins has increased globally and has led to an increased prevalence of (multi)vitamin supplement use (4, 5). In the US general population, for example, it was estimated that approximately one-third of adults were using multivitamin and-mineral supplements in 2012 (5). These supplements are part of a multibillion dollar dietary supplement industry that involved a whopping 40 billion dollar sale in 2016, in the US market alone (6).

Studies that have investigated the effects of vitamin supplementation on incidence and burden of various NCDs, have yielded variable results, with some of the studies observing a beneficial effect, and others reporting null or even harmful effects of vitamin supplementation (7-11). The latter two types of outcome have generated some skepticism regarding their use (12), which may have been one of the underlying causes for the observed slight decrease in the use of multivitamin and -mineral supplements in the US general population in the period of 1999-2012 (5).

Furthermore, the discordant findings of such studies make it difficult to draw meaningful conclusions based on single studies and may therefore necessitate combined analysis and interpretation of the data, as is done in meta-analyses.

While the rationale for vitamin supplementation may in some cases be valid, it is important to realize that many vitamins have an optimal range in which intracellular vitamin availability is sufficient for optimal enzymatic efficiency but does not exceed toxicity limits. In the future, it could be possible that biomarkers of vitamin status and particularly of vitamin functionality during use of vitamin supplements, will ‘guide’ dosing
regimens based on personalized needs. Such biomarker-guided personalized vitamin supplementation will not necessarily pertain to research settings, but will also likely find merit in other settings, such as professional sports and routine medical patient care. This approach would be desirable for every supplemented vitamin that is able to cause toxicity. However, it has not been well-documented which vitamins could be potentially toxic, since chronic vitamin toxicity often culminates in vague and ambiguous symptoms and therefore goes by unrecognized and unreported (13). Importantly, however, for many vitamins, measurement of the vitamin itself (i.e. direct biomarker, usually measured in the circulation) may not always reliably estimate true vitamin status, which is most precisely defined as the bioavailability of a vitamin at its active sites. These active sites are typically present intracellularly, and therefore efforts should be directed towards developing and validating functional biomarkers for every vitamin that can cause clinically relevant deficiency or toxicity.

One of the rationales for using vitamin supplements, is to alleviate symptoms of less clearly defined pre-existing conditions, such as FM and CFS. Accordingly, it has been shown that use of nutritional supplements is higher in these patients (35-68%) compared to the general population (27-56%) (14-18), and in some cases may lead to dangerously high vitamin intakes. However, the available evidence for a role of vitamins in the pathophysiology of FM and CFS is inconclusive. Moreover, it is not known whether vitamin supplementation has any effect on the spectrum of symptoms associated with FM and CFS.

A role for vitamins and minerals in fibromyalgia and chronic fatigue syndrome?

In chapter 1, we performed a systematic review with quality assessment to provide the first systematic overview of the existing literature on vitamins and minerals in the NCDs FMS and CFS. Moreover, by performing a meta-analysis of the included observational and intervention studies, we aimed to assess whether the available evidence supports a role for vitamins and minerals in the pathophysiology of FMS and CFS that could justify the frequent use of micronutrient supplements in this population.

Our findings from chapter 1 confirmed that the existing data, in particular data from randomized controlled trials, regarding many vitamins and minerals are scarce and conflicting. While no included study assessed vitamin B6 status, sufficient numbers of studies concerning vitamins C, D, and
E, calcium, and magnesium were included for meta-analysis. Only the data for vitamin E reached statistical significance, implying that vitamin E could be of importance in these disorders. Importantly, however, the outcome for vitamin E was significantly influenced by publication bias and thus might not have been truly significant. In general, the number of RCTs was alarmingly low and, despite adhering to our inclusion criteria, the included studies were of poor quality. The need for high-quality studies to provide more conclusive and mechanistic data on the potential role of vitamins and minerals in FMS and CFS, therefore remains.

In addition to alleviating of symptoms of pre-existing diseases, vitamin supplements are also consumed with the desire to prevent, or at least delay, the onset of age-related diseases, such as cardiovascular disease. Cardiovascular disease has been associated with many different vitamin deficiencies (19-21), including vitamin B6 (22). However, data regarding this association are conflicting in the general population, and are absent in other more specific populations, such as renal transplant recipients.

**Cardiovascular Aspects of Vitamin B6: Implications for the General Population?**

Early studies that demonstrated the detrimental effects of severe vitamin B6 deficiency on the cardiovascular system in monkeys (23, 24), have motivated a plethora of researchers to scrutinize the cardiovascular implications of vitamin B6 deficiency in humans. Constrained by ethical, financial, and technical issues, researchers were largely limited to observational studies, in which they had repeatedly observed strong inverse associations between plasma PLP and risk of adverse cardiovascular outcome (25-31). However, progressive insights into this topic have led to the belief that the association between a low plasma PLP concentration and a high risk of cardiovascular disease may be secondary to inflammation (32, 33). Unfortunately, data regarding a potential effect of inflammation were conflicting and were difficult to extrapolate to the general population. Therefore, in chapter 2, we comprehensively assessed the potential effect of inflammation on the association of plasma PLP with cardiovascular disease in a large general population-based prospective cohort.

We found that the association between plasma PLP and cardiovascular disease, defined as the composite of non-fatal and fatal cardiovascular events, was not independent of inflammation in the overall cohort. However, while plasma PLP concentration was similar between men and women, we observed
that the prospective association between plasma PLP and cardiovascular disease was significantly modified by gender. Our data inferred that plasma PLP is inversely associated with risk of cardiovascular disease in women, but not in men, independent of potential confounders such as inflammation. Albeit it is well known that there are great differences between sexes in terms of susceptibility to disease and treatment (34), little is known about potential sex-specific effects of vitamin B6. To the best of our knowledge, the current evidence for sex-specificity of vitamin B6 is provided by two studies. A rat study performed back in 1965, showed that vitamin B6 deficient female rats on a high-cholesterol diet were more prone to developing severe aortic lesions, compared to vitamin B6 deficient male rats (35). More recently, a metabolomics study by Krumsiek et al aiming at characterizing metabolic differences between sexes, showed and replicated a significant sex-specific difference in vitamin B6 metabolism (36). They revealed that vitamin B6 metabolism was significantly higher in males compared to females (P-value=7x10^{-13}), but the researchers did not discuss potential implications of this difference. However, a more active vitamin B6 metabolism in males could potentially explain the findings from the 1965 rat study and thus possibly indicate that vitamin B6 deficiency could disproportionally increase susceptibility to cardiovascular disease in women, compared to men. This hypothesis fits with our data from chapter 2, where the association between plasma PLP and cardiovascular disease was stronger in women, compared to men. Importantly, Krumsiek et al justly noted that the statistical significance does not equal biological relevance and that more studies are necessary to investigate in more detail the potential clinical implications of sex-specific differences in vitamin B6 metabolism in humans, as we found in chapter 2. For this, conclusive data should preferably be obtained from carefully performed, biomarker-guided, intervention studies.

Prevalence, Functional Effects, and Clinical Implications of Vitamin B6 Deficiency in Renal Transplant Recipients

Chronic kidney disease (CKD) poses a major global disease burden, as it affects up to 16% of the general population (37). CKD patients suffer from a progressive loss of kidney function, which culminates in end-stage renal disease (ESRD). For the majority of ESRD patients, the renal replacement therapy of choice is renal transplantation, resulting in marked improvements in quality of life and life expectancy compared to dialysis (38, 39). While improved pharmacotherapy and surgical techniques have markedly improved
short-term survival, considerable improvements on the long-term remain to be achieved (40). With 10-year mortality rates ranging from 24% for living-donor recipients to 36% for deceased-donor recipients, RTR remain at increased risk of mortality compared to the general adult population (10-year mortality rates <1%) (41). In RTR, cardiovascular disease is the major cause of death, accounting for approximately 28% of deaths (41, 42). Altogether, these data reflect the need for non-conventional strategies to ameliorate cardiovascular outcome and improve long-term survival of recipients. In light of this, we assessed plasma PLP in a large cohort of stable RTR with long-term follow-up in chapter 3. We demonstrated that vitamin B6 deficiency is prevalent in RTR, despite adequate intake of vitamin B6 in this population. It thus seems that renal insufficiency, or a related factor, adversely influences the handling of vitamin B6 in humans. Future studies should attempt to identify the mechanisms behind these observations. In addition, we showed that vitamin B6 deficient RTR were at increased risk of cardiovascular mortality, compared to vitamin B6 sufficient recipients. In chapter 4, we investigated whether vitamin B6 functionality was affected in vitamin B6 deficient RTR, by measuring the functional vitamin B6 marker plasma 3-HK/XA ratio. Indeed, we found that vitamin B6 deficient RTR had a significantly lower functional vitamin B6 status compared to vitamin B6 sufficient RTR. Importantly, a low functional vitamin B6 status was independently associated with increased risk of mortality.

**Chapters 3 and 4** collectively show that vitamin B6 deficiency has functional and long-term clinical implications after renal transplantation. However, while 3-HK/XA ratio has several important advantages over plasma PLP, e.g. a weaker association with inflammation, it is not without drawbacks. Both 3-HK and XA are hydrophilic compounds that are eliminated from the circulation by the kidneys. An impaired kidney function could therefore influence circulating concentrations of these compounds and thus result in a biased 3-HK/XA ratio. Our data from chapter 4 support the possibility of bias by renal function, as they revealed a positive association between the ratio and eGFR. Notably, the association between eGFR and XA was much stronger compared to that with 3-HK, indicating that bias from renal function may be due to an easier elimination of XA, compared to 3-HK.

It would be important to distinguish whether the observed prospective associations are causal or whether vitamin B6 deficiency is merely a risk factor of an unfavorable risk profile. If vitamin B6 deficiency is causally related with outcome, it could serve as a modifiable factor to improve cardiovascular
outcome and survival in RTR. Promisingly, pyridoxine-supplementation in patients with suspected coronary artery disease, not only increased plasma PLP concentration, but also resulted in an improvement of functional vitamin B6 status, especially in patients with low plasma PLP concentrations at baseline (43).

The Kynurenine Pathway after Kidney Transplantation

Vitamin B6 deficiency and chronic low-grade inflammation are prevalent in RTR and have both been associated with adverse long-term outcome (44). Since these conditions are able to influence activities of kynurenine pathway enzymes (45, 46), it is likely that the flow of metabolites through this pathway is disturbed in many RTR. It has long been known that the kynurenine pathway constitutes several highly bioactive compounds, e.g. 3-HK (47), and it is this conceivable that a disturbed kynurenine pathway may have clinical implications on the long-term. Strikingly, however, these implications had not been fully evaluated in RTR (48-51). Therefore, in chapter 5, we studied the potential clinical implications of kynurenine pathway activation in RTR. To this end, we assessed the potential relationships of Trp, Kyn, 3-HK, IDO-activity, and KMO-activity, with long-term graft failure and all-cause mortality in a large prospective cohort of stable RTR. Our main findings were that the assessed kynurenine pathway parameters were consistently associated with the different inflammation parameters and that higher plasma concentrations of Kyn, 3-HK, and higher activities of IDO and KMO were independently associated with increased long-term risk of graft failure. Moreover, we found higher plasma 3-HK concentration to be associated with increased risk of long-term all-cause mortality in RTR. Although 3-HK had not previously been studied in the context of renal transplantation, our data are in line with neurological studies which revealed that 3-HK was able to induce cell death by causing formation of reactive oxygen species and by impairing cellular energy metabolism (52, 53). The cytotoxic effects 3-HK might be of particular importance in RTR with low functional vitamin B6 status, as inflammation and vitamin B6 deficiency may synergistically increase plasma 3-HK concentration by increasing the flow of Trp to 3-HK and by impairing metabolism of 3-HK to either XA or HAA, respectively. However, the impact of vitamin B6 deficiency on the kynurenine pathway in RTR is yet to be assessed.

While clearly indicating the clinical relevance of the kynurenine pathway, our data pose several limitations that are worth considering. For example, it is
difficult to ascertain to what extent the Trp/Kyn and 3-HK/Kyn ratios reflect the appropriate enzyme activities and thus it remains uncertain whether the increased risk of graft failure is related to the enzyme functions or whether it could be ascribed to the biological functions of Trp, Kyn, or both. Similarly, the putative adverse effects of 3-HK on graft function and survival may not necessarily be due to the cytotoxic effects of 3-HK, but instead may be a reflection of functional vitamin B6 deficiency. Previous studies have indeed shown that plasma 3-HK increases in vitamin B6 deficient individuals. However, due to the strong associations of 3-HK with inflammation markers and kidney function, as observed by others (43, 54) and our group in chapter 5, plasma 3-HK concentration is nowadays considered a less reliable marker of functional vitamin B6 status compared to, for example, the 3-HK/XA ratio in plasma (55).

**General Conclusions**

Based on findings of this thesis, we conclude that the available data for a role for vitamins and minerals in the pathophysiology of FMS and CFS are derived from poor-quality studies and are conflicting. These data do not support such a role for vitamins or minerals and, therefore, do not justify the use of micronutrient supplements to alleviate FMS and CFS symptoms. Furthermore, a low (functional) vitamin B6 status is independently linked to adverse long-term outcome in women and RTR. In RTR, Trp metabolism along the kynurenine pathway seems disturbed, which might give rise to an disadvantageous profile of bioactive kynurenines and thus, especially though increased levels of 3-HK, might adversely affect long-term graft function and survival.

**Future Perspectives**

Vitamin research is moving forward and continues to provide new useful insights into the relevance of vitamins for a healthy and longer life. While this thesis has contributed to our knowledge on vitamins, in particular vitamin B6 and related pathways, many aspects are yet to be elucidated.

For example, we have shown that vitamin B6 deficiency in RTR is not due to inadequate vitamin B6 intake or the presence of chronic low-grade inflammation, but we do not know which factors are responsible for this observation. There are several hypotheses that may explain our findings. First, vitamin B6 deficiency in RTR may be caused by impaired absorption
of vitamin B6 in the small intestine. This hypothesis might be relevant, since RTR frequently suffer from gastrointestinal complications as a side effect of commonly used immunosuppressive and antibiotic drugs and as a consequence of infection with pathogenic microorganisms (56). Second, it is possible that vitamin B6 deficiency in RTR is due to increased catabolism of PLP to PA, as observed during systemic inflammation (57). These hypotheses could be investigated in an intervention study involving vitamin B6 deficient recipient. The study design would preferable involve administration of labeled vitamin B6 and repeated collection of blood, urine, and faeces, to comprehensively assess the metabolic fate of ingested vitamin B6 in RTR. Vitamin B6 catabolism could in that case be estimated via the PAr index, which is calculated from the concentrations of pyridoxic acid (PA), PLP, and pyridoxal (PL) in plasma (57).

In addition, plasma levels of kynurenines depend highly on kidney function (58, 59). Since kidney function is thus likely to confound interpretation of substrate product ratios of enzymes in the kynurenine pathway as indicators of functional vitamin B6 status, it would be important to validate these ratios in a population with impaired kidney function, e.g. RTR.

Furthermore, the potential long-term implications of many kynurenines remain uninvestigated in the context of renal transplantation. Examples of potentially relevant bioactive kynurenines, include the neuroprotective kynurenic acid, the neurotoxic N-methyl-D-aspartate receptor agonist quinolinic acid, and the relatively unknown picolinic acid (60, 61). Also, it is conceivable that activation of the kynurenine pathway, which is likely the case in RTR, might deprive circulating Trp levels and thereby affect levels of other bioactive Trp metabolites, such as melatonin and serotonin (62-65). Therefore, it would also be important to assess a potential link between inflammation, Trp metabolism, mood, and sleep in RTR.

To further increase our knowledge on the (patho)physiological relevance of vitamins, future studies will rely on innovations in the area of clinical chemistry. Through development of more sensitive and comprehensive laboratory assays, as seen with modern liquid chromatography mass spectrometry assays, these innovations will enable assessment of the effects of vitamin insufficiencies and deficiencies on the flow of a related biochemical pathway, thereby likely providing a better understanding of the functionality of a vitamin, compared to measurement of one pathway constituent or of the vitamin itself. The resulting development of functional biomarkers, like the 3-HK/XA ratio for vitamin B6, would not only provide information on the
need for supplementation, but would also enable biomarker-guided titration of vitamin supplementation. Such functional biomarkers would be a useful addition to vitamin supplementation studies, and could also be of added value in preventing toxicity in individual cases, such as that of Sven Kramer. However, it is important to note that functional biomarkers may also be affected by various factors, such as kidney function in the case of the 3-HK/XA ratio, and that they should be interpreted with caution in individuals with abnormalities in these factors. Identification of possible interferences should therefore be a vital part of validation during development of biomarkers of functional vitamin status. In conclusion, the vast and continuously growing body of evidence in support of potential health benefits of an adequate vitamin B6 status, should prompt researchers to further identify biochemical pathways, types of pathophysiology, and populations that might be affected by an inadequate vitamin B6 status. Moreover, this evidence should motivate the scientific community to address the need for long-term intervention studies with biomarker-guided personalized vitamin B6 supplementation that will accommodate assessment of the potential implications of correcting vitamin B6 deficiency in susceptible populations, such as women and RTR, without abandoning safety.
References


6. The $37 billion supplement industry is barely regulated — and it's allowing dangerous products to slip through the cracks. [updated 2017/11/08; ]. Available from: https://www.businessinsider.nl/supplements-vitamins-bad-or-good-health-2017-8/?international=true&r=US.


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