Direct and Functional Biomarkers of Vitamin B6 Status

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Keywords

Abstract

Measures of B6 status are categorized as direct biomarkers and as functional biomarkers. Direct biomarkers measure B6 vitamers in plasma/serum, urine and erythrocytes, and among these plasma pyridoxal 5′-phosphate (PLP) is most commonly used. Functional biomarkers include erythrocyte transaminase activities and, more recently, plasma levels of metabolites involved in PLP-dependent reactions, such as the kynurenine pathway, one-carbon metabolism, transsulfuration (cystathionine), and glycine decarboxylation (serine and glycine). Vitamin B6 status is best assessed by using a combination of biomarkers because of the influence of potential confounders, such as inflammation, alkaline phosphatase activity, low serum albumin, renal function, and inorganic phosphate. Ratios between substrate-products pairs have recently been investigated as a strategy to attenuate such influence. These efforts have provided promising new markers such as the PAr index, the 3-hydroxykynurenine:xanthurenic acid ratio, and the oxoglutarate:glutamate ratio. Targeted metabolic profiling or untargeted metabolomics based on mass spectrometry allow the simultaneous quantification of a large number of metabolites, which are currently evaluated as functional biomarkers, using data reduction statistics.

Contents

INTRODUCTION

In 2001, the National Institutes of Health (NIH) Biomarkers Definition Working Group proposed the definition of biomarker as "a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (16, p. 91). Nutritional biomarkers as opposed to diagnostic laboratory tests (117) often are not linked to a specified outcome but rather reflect a continuous trait, i.e., nutritional status. They are categorized into three groups that serve as (*a*) means of validating of

dietary instruments, (*b*) surrogate indicators of dietary intake, or (*c*) integrated measures of nutritional status, or any combination of these utilities (175). Measurement of a nutritional biomarker allows the assessment of nutritional status reflecting food intake, supplement use, bioavailability, metabolism, and excretion (172).

Dietary instruments such as the 24-hour dietary recall and/or food frequency questionnaires to assess habitual food intake represent the cornerstone for the evaluation of nutritional biomarkers (135). Understanding sources of variability, such as demographic factors, diseases, drugs, and lifestyle factors including alcohol use and smoking, is important for optimal use and interpretation of data on nutritional biomarkers (174). These factors may influence the within-subject and/or between-subject variability of a biomarker. It is therefore critical, particularly for assessment on an individual basis, to determine whether a single biomarker measurement reflects the exposure over time. Within-person reproducibility can be expressed as the intraclass correlation coefficient (ICC), which is the between-person variance divided by the sum of the within- and between-person variance. An ICC of <0.40 indicates poor reproducibility, 0.40 to 0.75 indicates fair to good reproducibility, and >0.75 indicates excellent reproducibility (190). The ICC has been obtained for some commonly used B6 biomarkers (45, 147), but, regrettably, such data are lacking for most biomarkers.

Biomarkers of vitamin B6 status are categorized as direct or functional indices. Direct indices measure the concentration of B6 forms in the circulation or urine, whereas functional indices reflect the metabolic effects of vitamin B6 serving as an enzyme cofactor (18, 110). This review covers direct and functional B6 biomarkers and addresses some novel biomarkers based on metabolic profiling and metabolite ratios.

VITAMIN B6 FORMS AND FUNCTION

Vitamin B6 is a generic term that refers to six interconvertible compounds that share a 2-methyl-3-hydroxypyridine structure with variable substituents at positions C4 and C5, i.e., pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL), and their phosphorylated derivatives pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP), and pyridoxal 5′-phosphate (PLP) (**Figure 1**). PLP is the coenzyme form of vitamin B6 that serves as cofactor in more than 160 different catalytic functions, including transaminations, aldol cleavages, α-decarboxylations, racemizations, β and γ eliminations, and replacement reactions. Most PLP-dependent enzymes are involved in amino acid biosynthesis and degradation. They have an important role in the metabolism of neurotransmitters, such as dopamine, serotonin, glycine, glutamate, and γ-aminobutyric acid (GABA), but also organic acids, glucose, sphingolipids, and fatty acids (56, 171). There has been interest in potential therapeutic uses of vitamin B6 analogues for many years, along with a more recent focus on certain PLP-dependent pathways as potential targets for drug development or for being linked to pathological processes. Examples of drug targets are DOPA decarboxylase, GABA aminotransferase, and serine hydroxymethyltransferase (5). Tryptophan catabolism via the kynurenine pathway (**Figure 2**) involves several PLP-dependent reactions that produce neuroactive and immunomodulating compounds (27, 40). Two PLP-dependent enzymes of the transsulfuration pathway (**Figure 3**) convert homocysteine to cysteine, thereby linking one-carbon metabolism to synthesis of glutathione and the vasodilator gas, hydrogen sulfide (113). In addition to its function as a cofactor, PLP has recently been suggested as a scavenger of reactive oxygen species (53, 95), and an oxidative stress response in tissues also appears to be a consequence of vitamin B6 deficiency (115).

The production of PLP in liver is regulated (144) such that PLP levels in liver remain relatively constant even at very high intake of PN (197). Liver is the organ responsible for the formation
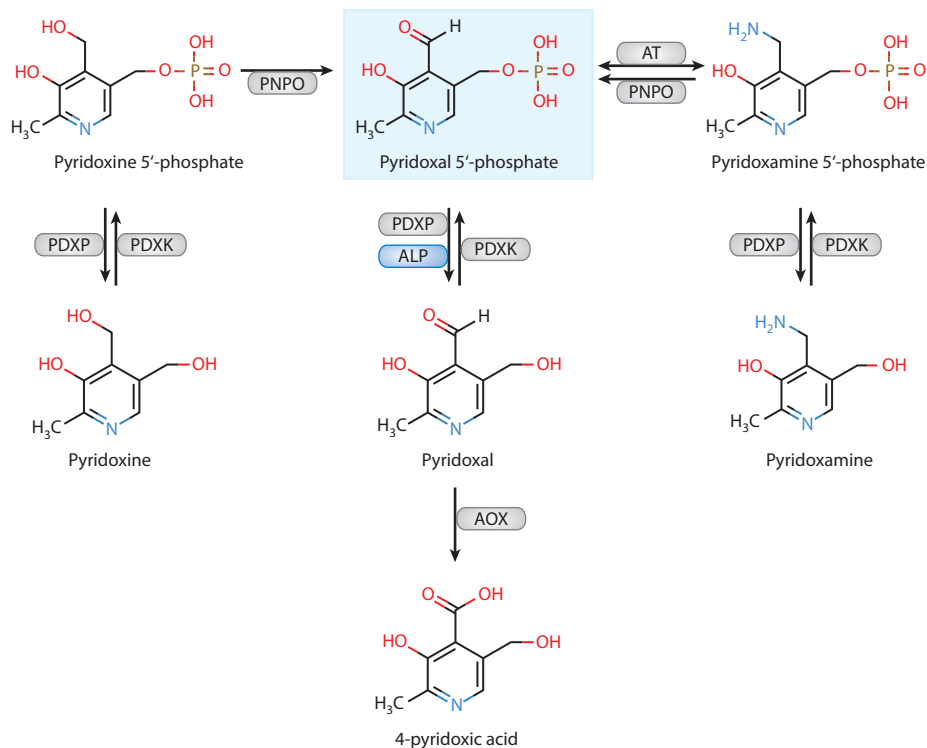
Figure 1

Vitamin B6 species, their metabolism, and the enzymes involved. Pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM) are phosphorylated by pyridoxal (pyridoxine, vitamin B6) kinase (PDXK; EC 2.7.1.3511) to pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP), and pyridoxamine 5′-phosphate (PMP), respectively. Pyridoxamine 5′-phosphate oxidase (PNPO; EC 1.4.3.5) oxidizes PNP and PMP to PLP. In the liver, PLP bound to albumin is exported into the circulation for delivery to tissues. Before cellular uptake, extracellular PLP is dephosphorylated to PL by the ectoenzyme, tissue-nonspecific alkaline phosphatase (ALP; EC 3.1.3.1). The PLP-to-PL conversion is also catalyzed by the intracellular enzyme, pyridoxal (pyridoxine, vitamin B6) phosphatase (pyridoxal phosphate phosphatase, PDXP; EC 3.1.3.74). The formation of the vitamin B6 catabolite, 4-pyridoxic acid (PA), from PL is catalyzed by aldehyde oxidase(s) (AOX). Abbreviation: AT, aminotransferase.

of PLP in plasma (83, 129), which in addition to PLP (about 70–90%) also contains PL (8–30%) and 4-pyridoxic acid (PA), a vitamin B6 catabolite that is excreted into the urine (110, 213). The concentration of plasma PLP in an individual is increased with vitamin B6 intake over a broad range (75, 76). In plasma, PLP is strongly bound to albumin (83, 129), and free PLP can be dephosphorylated to PL in a reaction catalyzed by tissue-nonspecific alkaline phosphatase (ALP). This is an ectoenzyme located on the outer membrane of cells, including erythrocytes (26). Plasma PL is derived from several organs and is the transport form capable of crossing biological membranes; it can thereby be taken up by tissues (130).

VITAMIN B6 AND HEALTH

Although certain patterns of food selection can contribute to inadequate intake, overt vitamin B6 deficiency caused by dietary insufficiency is rare in developed countries because vitamin B6 is present in most foods. Isolated deficiency is uncommon, and it usually occurs in combination
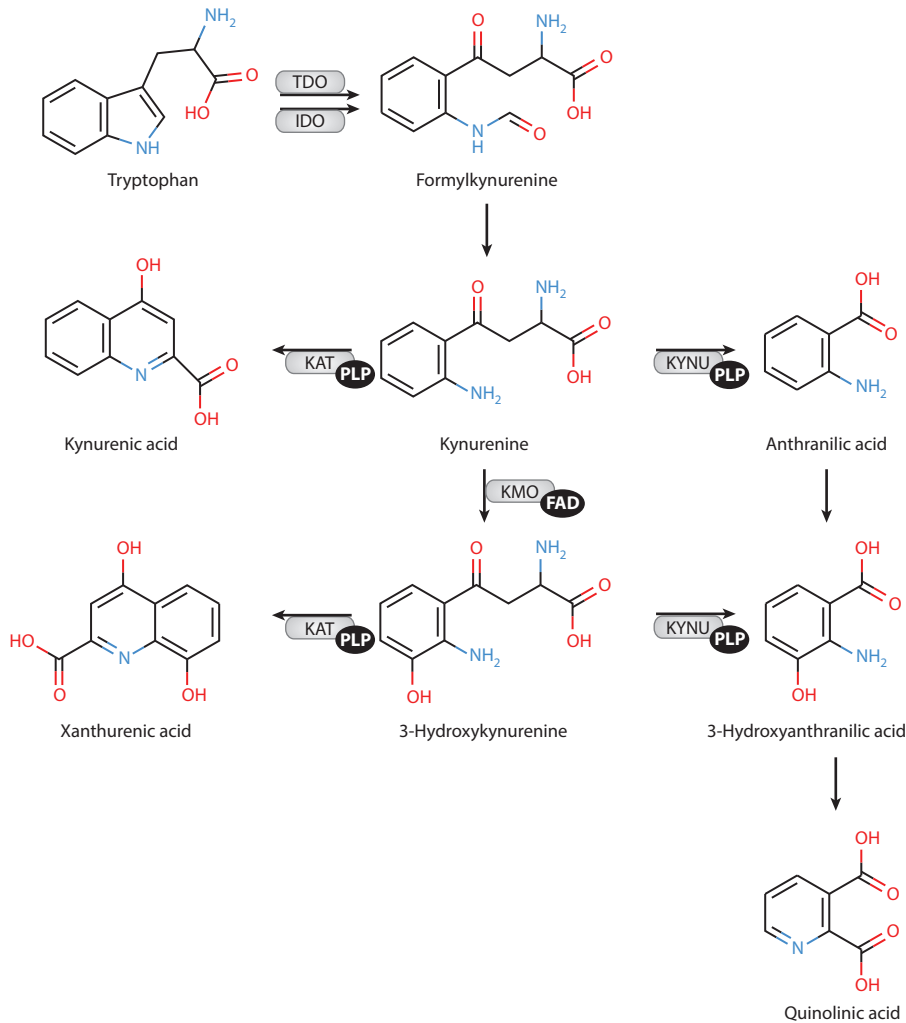
Figure 2

The kynurenine pathway of tryptophan catabolism and the enzymes and cofactors involved. The heme dioxygenases, hepatic tryptophan (2,3)-dioxygenase (TDO; EC 1.13.1.2) and ubiquitous indoleamine (2,3)-dioxygenase (IDO; EC 1.13.11.42), catalyze the oxidation of L-tryptophan to N-formylkynurenine, which is the first and rate-limiting step of tryptophan catabolism. IDO is activated by proinflammatory cytokines such as interferon-gamma and tumor necrosis factor-alpha (101, 107). *N*-formylkynurenine is rapidly converted by formamidase (not shown) to kynurenine (Kyn). Kyn is converted to 3-hydroxykynurenine (HK) by flavin adenine dinucleotide (FAD)-dependent kynurenine mono-oxygenase (KMO; EC 1.14.13.9) and then cleaved to 3-hydroxyanthranilic acid by the pyridoxal 5′-phosphate (PLP)-dependent enzyme kynureninase (KYNU; EC 3.7.1.3), which also catalyzes the conversion of Kyn to anthranilic acid. The PLP-dependent enzyme kynurenine transaminase (KAT) catalyzes the formation of two end-stage metabolites, kynurenic acid (from Kyn) and xanthurenic acid (from HK).

Figure 3

The transsulfuration pathway. Homocysteine is formed by hydrolysis of *S*-adenosylhomocysteine, which is formed from *S*-adenosylmethionine during transmethyl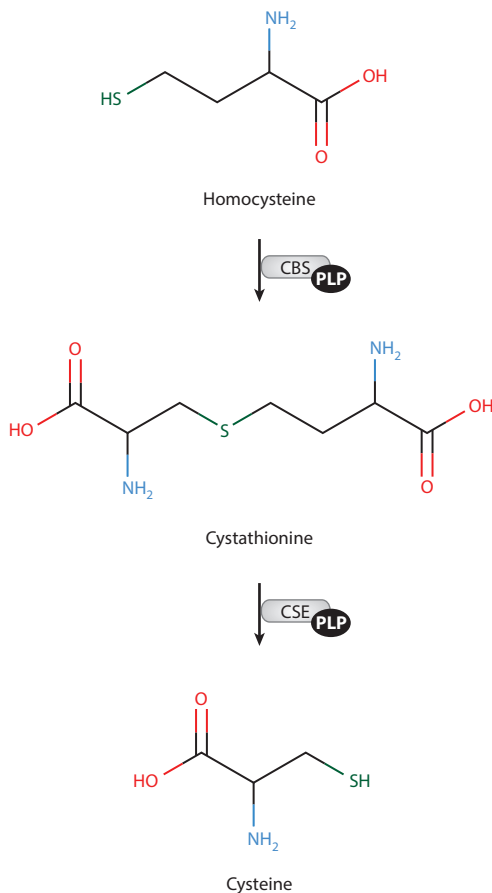ation reactions (not shown). Homocysteine is either remethylated to methionine (not shown) or converted to cysteine via the transsulfuration pathway, where homocysteine is converted to cysteine through the sequential action of two pyridoxal 5′-phosphate (PLP)-dependent enzymes, cystathionine beta-synthase (CBS; EC 4.2.1.22) and cystathionine gamma-lyase (CSE; EC 4.4.1.1).

with deficiencies of other B vitamins. Low B6 status has been reported in contraceptive users, smokers, alcoholics, and patients with celiac disease or diabetes (53, 210). Secondary vitamin B6 deficiency may result from mutations causing defects in B6 salvage pathways, inborn errors causing accumulation of intermediates that react with PLP, and intake of drugs that reduce the availability of PLP (41).

In the general population, low vitamin B6 intake is associated with an increased risk of age-related diseases, such as cardiovascular disease (182, 216) and cancer (100, 114, 218, 246). However, conflicting results have been obtained on B6 intake and disease risk (105, 255).

Plasma PLP concentration is not strongly related to estimated vitamin B6 intake in population studies (105, 124, 182). Limitations in the estimation of vitamin B6 intake in population studies certainly confound studies of diet–disease relationships and associations of dietary intake with

biomarkers of B6 status (178). The incomplete and variable bioavailability of many dietary sources of vitamin B6 (69–71) further complicates studies of dietary vitamin B6 and disease incidence.

Low plasma PLP has been associated with an increased risk of diseases such as cardiovascular disease (119), stroke (99), and cancer (91). However, such diseases may be linked to increased ALP, hypoalbuminemia, and inflammation, which may confound the interpretation of the observed associations, as detailed below.

VITAMIN B6 AND INFLAMMATION

Low plasma PLP has been observed in a variety of conditions including rheumatoid arthritis (RA) (36, 84, 191), inflammatory bowel disease (IBD) (201), cardiovascular diseases including stroke (99, 119), diabetes (64), deep venous thrombosis (29, 193), and cancer (38). These are mostly age-related diseases where inflammation is involved in pathogenesis (77, 240). In both RA and IBD patients, plasma PLP is inversely related to the severity of the disease (34, 193). In RA patients, there are no associations between vitamin B6 intake and plasma PLP, no indication of congenital defects of vitamin B6 metabolism (34, 35, 168), and no vitamin B6 deficiency as judged by other B6 markers, such as erythrocyte aspartate transaminase activity coefficient (EAST-AC) (191), erythrocyte PLP (35), or urinary PA excretion (35). No change in urinary PA excretion suggests that increased vitamin B6 catabolism does not explain altered B6 homeostasis during chronic inflammation (36).

Plasma PLP shows an inverse relation to C-reactive protein (CRP), other inflammatory markers (194), and acute-phase reactants (12) in population-based cohorts (66, 155, 204) as well as in clinical studies (64, 65, 85, 193, 230).

In critically ill patients, plasma PLP and plasma PA, but not vitamin B6 intake and EAST-AC, are significantly associated with immune response (85). The association between plasma PA and immune response suggests that vitamin B6 catabolism is involved during the acute phase (85). In critically ill patients with systemic inflammation, vitamin B6 supplementation is associated with no or a moderate increase in plasma PLP as compared to a 15- to 20-fold increase in PL (33) and 3-fold increase in erythrocyte PLP (176), suggesting increased cellular uptake of vitamin B6.

Low plasma PLP in inflammatory conditions parallels a reduction in liver but not muscle PLP and may reflect tissue-specific mobilization of this cofactor to the sites of inflammation, a hypothesis that has been supported by experimental studies in rats (36, 237). This apparent alteration in PLP distribution during inflammation highlights the importance of combining multiple biomarkers for the assessment of vitamin B6 status in clinical and epidemiological studies, as emphasized by Leklem 25 years ago (110).

DIRECT INDICES OF VITAMIN B6 STATUS

Direct indices or measures refer to concentrations of B6 vitamers in the circulation (plasma or erythrocytes) or urine.

Plasma Pyridoxal 5′-Phosphate

Plasma PLP seems to reflect the PLP content in liver but not PLP content in muscle (36, 130), which is resistant to B6 depletion (44). It shows a positive association with vitamin B6 intake (13, 75, 76, 87) and responds about tenfold to supplementation (19) and within 1–2 weeks to B6 depletion (51, 104) and repletion (87, 102, 110).

Plasma PLP declines in samples stored at room temperature (89, 165) and in samples exposed to light (236), but it is fairly stable at low temperature (147) and in frozen samples (89). It has a sufficiently high within-person reproducibility over years (ICC of about 0.65) that allows one-exposure assessment of vitamin B6 status (45, 147).

Plasma PLP decreases within hours after glucose ingestion or high carbohydrate intake (112) and shows an inverse association with fasting glucose and HbA1c (204). These observations suggest that fasting blood samples should be measured when assessing vitamin B6 status but also indicate that high blood glucose in diabetics should be taken into account when evaluating plasma PLP concentration.

Plasma PLP is the most commonly used indicator of vitamin B6 status (110). However, several factors not related to vitamin B6 status affect plasma PLP. In addition to inflammation, these include albumin concentration, alkaline phosphatase activity, and alcohol consumption, which (together with vitamin B6 intake) explain 30–40% of the variance in plasma PLP (24).

Plasma PLP is bound to and is positively associated with serum albumin (36, 129, 237), which decreases during the acute phase (208). Low PLP in RA patients with moderate inflammation (83) and in critically ill patients (85) has been attributed to reduced binding of PLP to albumin. Because of high protein binding of PLP in plasma, measurement of PLP in undeproteinized serum/plasma gives false low PLP concentrations (179). Thus, methods that have been proposed to measure PLP in plasma by direct analysis (i.e., without deproteinization) are suspect, at best.

Plasma PLP is inversely associated with tissue-nonspecific ALP (12, 36, 90, 247). The association is particularly strong among the elderly (12). The importance of ALP as a determinant of plasma PLP (58) is demonstrated by inborn errors involving change in enzyme activity. In patients with hypophosphatasia characterized by little or no plasma ALP activity caused by mutations in the *ALPL* gene (153), plasma PLP is massively increased above reference values (90, 248). On the other end of the spectrum, children with familial hypophosphatemic rickets have elevated ALP and extremely low plasma PLP (15 out of 31 children had a level less than 0.2 nmol/L) (181).

Plasma PLP has been shown to be associated with up to 20 single-nucleotide polymorphisms (SNPs) in the ALPL gene in genomewide association studies (28, 79, 98, 215) as well as in candidate gene analyses (28). Some ALPL SNPs related to plasma PLP are associated with ALP activity in the direction accounting for change in PLP (28, 79, 215), whereas PLP associations for two SNPs are attenuated when ALP is included in the regression model (215). These observations suggest that the associations of ALPL SNPs with plasma PLP are mediated by ALP activity. No ALPL SNPs are associated with plasma PL, PA, and the functional B6 marker, HK:XA ratio (28), indicating no functional changes in vitamin B6 status. Because most SNPs associated with plasma PLP have a minor allele frequency >0.10 (28), the genetic makeup may represent a common factor interfering with the assessment of vitamin B6 status based on PLP measurement.

Under physiological conditions, inorganic phosphate inhibits ALP by up to 50%, which may indirectly impact plasma concentration of PLP (42). The inhibition may explain the positive correlation between plasma PLP and inorganic phosphate (139) and the increased plasma PLP in postmenopausal women with elevated serum inorganic phosphate inherent to bone loss (139). Low plasma PLP has been noted in conditions with elevated ALP, such as liver and bone disease, cancer, pregnancy (6, 11, 143), and diabetes (low PLP:PL ratio) (138, 161); plasma PLP is essentially unaffected by renal function (12).

In conclusion, plasma PLP reflects vitamin B6 intake and the liver PLP pool. Because of the influence of inflammatory conditions, serum albumin and ALP, treatment with some drugs (53), and changes during pregnancy, its validity as a status indicator in a diseased population has been questioned (110, 237). However, in healthy, nonpregnant subjects, the use of plasma PLP as a status indicator seems to be appropriate (76).

Plasma Pyridoxal and Total B6-Aldehyde (PLP + PL)

Plasma PL, the transport form of vitamin B6, has been suggested as an additional marker of vitamin B6 status (110). From a contemporary analytical point of view, PL is easy to obtain, since it is determined together with other B6 vitamers by high-performance liquid chromatography (HPLC) techniques (17, 192, 225) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (145). Plasma PL shows a strong correlation with PLP (Spearman r of 0.51–0.80) (19, 149), responds within weeks to depletion and repletion (87), and increases even more than plasma PLP following supplementation with pyridoxine (19, 217, 253), but less than PLP in response to dietary B6 intake (76). Pharmacokinetic studies have shown that plasma PL responds rapidly following a single oral dose of a multivitamin supplement containing PN [time to maximal concentration (Tmax) $\sim$1 h], whereas PLP exhibits a Tmax of $\sim$10 h (217).

The preanalytical stability of PL varies according to sample matrix, which probably reflects formation from PLP, degradation, and cellular uptake by blood cells, and may explain the wide variability in plasma PL concentrations reported in the literature (19). PL is relatively stable in ethylenediaminetetraacetic acid (EDTA) plasma (89); decreases in samples exposed to light (236); increases markedly in serum, heparin plasma, and citrate plasma (at room temperature) (89); but declines markedly in chilled whole blood (147). Such variability may partly explain why the reported within-person reproducibility (ICCs) of plasma PL over years varies between studies and populations from 0.17 (45) to 0.61 (147) and why PL does not capture moderate changes in vitamin B6 status according to dietary vitamin B6 intake (76).

During prolonged exposure to room-temperature conditions, PLP declines in plasma/serum due to dephosphorylation to PL. The loss of PLP is quantitatively recovered as PL, and total B6-aldehyde (PLP + PL) is relatively stable. Thus, summarizing the concentrations of PLP plus PL has been suggested as a strategy to correct for partial degradation of PLP during nonoptimal sample handling (89). Total plasma B6 ($\sim$90% of which is composed of PLP + PL) has also been measured by microbiological assay (110), and as PL after acid phosphatase treatment of plasma samples (137), and this value correlates with plasma PLP (106).

The plasma PLP:PL ratio is lower in critically ill patients with systemic inflammation in comparison with controls. Plasma PL shows a stronger correlation than plasma PLP with erythrocyte PLP (237). On the basis of these observations, the authors suggest that plasma PL may serve as a surrogate marker of intracellular PLP (237). Furthermore, the combined measurement of plasma PLP and PL may give additional information about distribution and homeostasis of B6 species in plasma, which are affected by altered ALP activity in vivo (11). Measurement of total B6-aldehyde has also been recommended as direct marker of B6 status in subjects/patients with increased ALP, such as pregnant women (11) and diabetic patients (138).

In conclusion, the advantages of measuring plasma PL or PLP + PL (110) are yet to be properly validated, and cut-off reference values have not been established (138).

Plasma 4-Pyridoxic Acid

PA is a vitamin B6 catabolite formed from PL in the liver. It has high renal clearance (254), which is about twice that of creatinine clearance (43). PA is not protein bound in plasma. It is measured together with PLP and PL by HPLC techniques (72, 192) and LC-MS/MS (145). It shows a strong correlation with plasma PLP (Spearman r of 0.52–0.67) and an even stronger correlation with plasma PL (Spearman r of 0.52–0.79) (19, 149). The PA-PL correlation (but not the PA-PLP correlation) actually increases considerably after supplementation with pyridoxine (19, 253). Plasma PA responds within 1–2 weeks to a change in vitamin B6 intake (13, 76) and

increases about 50-fold following supplementation with pyridoxine (40 mg daily) (19). PA is stable in EDTA, heparin, and citrate plasma and serum at room temperature (89), in samples exposed to light (236), in chilled whole blood (147), and in frozen samples (89), and it is not related to plasma ALP activity (12).

Plasma PA has variable within-person reproducibility, over years, that ranges from good in healthy postmenopausal women (ICC of 0.74), to fair (ICC of 0.42) in cardiovascular patients (147), and to poor in other populations (45). Since the preanalytical stability is good, variable reproducibility may partly reflect that plasma PA is strongly related to renal function (12) and increases severalfold in patients with renal dysfunction (43). Notably, in contrast to PLP, PA is not influenced by acute-phase inflammatory status in the general population (12) but is positively related to markers of cellular immune activation (230) and increases dramatically in critically ill patients (85), which suggests that vitamin B6 catabolism increases in severely diseased subjects.

Compared to PLP, plasma PA is influenced by other confounding factors (12), and has been suggested as a possible complementary (12) and short-term (192) marker of vitamin B6 status. However, the strong influence from kidney function decreases the specificity of plasma PA as a biomarker of vitamin B6 status.

Urinary Excretion of 4-Pyridoxic Acid and Other B6 Vitamers

PA represents >90% of vitamin B6 species excreted into the urine (126, 189). Approximately 40–60% of dietary vitamin B6 will be excreted as PA (110), with the excretion being inversely associated with protein intake (102) but also affected by the quality of protein (75).

Urinary PA varies according to vitamin B6 intake (75, 76) and responds within 1–2 weeks to vitamin B6 depletion and repletion (87, 102, 110). It shows a strong correlation with PA ($r = 0.51$) and PLP in plasma ($r = 0.77$) and erythrocyte PLP ($r = 0.73$) but a weak correlation with EAST-AC (43, 76).

Urinary PA responds almost immediately to change in dietary intake (126), in contrast to plasma PLP, which reflects vitamin B6 tissue saturation status (110). This view is based on results from kinetic studies showing that urinary PA and plasma PLP increased within days after starting four weeks of intravenous pyridoxine administration. When supplementation was terminated, PA excretion declined to presupplementation levels within days, whereas plasma PLP remained elevated for months (126). Thus, excretion is a measure of recent vitamin B6 intake and is a useful instrument for the assessment of vitamin B6 requirements (87). However, its use as marker of vitamin B6 status requires information on the recent individual vitamin B6 intake and diet (110) and has been discouraged (210).

The measurement of PA excretion in combination with markers reflecting metabolic alteration can provide important information for a more accurate evaluation of nutritional vitamin B6 status and intake. Other attractive features include the lack of influence from age (96), pregnancy (221), alcohol, and oral contraceptives (18, 23, 111). An excretion of PA greater than 3 μmol/day has been proposed as indicative of adequate status (110). For large-scale studies, collection of 24-hour urine is impractical, but urinary PA can be measured as the PA:creatinine ratio in random samples (199). However, urinary PA shows circadian variations (253), which may increase between-subject variance of PA excretion determined from spot urine. Another disadvantage is decreased excretion rate in subjects with inadequate riboflavin status (207).

Aside from PA, vitamin B6 appears mainly in the urine as pyridoxal and to a lesser extent as pyridoxamine (196). A 24-hour urine collection over one to three weeks has been recommended for nutritional status assessment when using total vitamin B6 excretion as an indicator (110). However, the use of random fasting samples and the determination of vitamin B6 expressed as per

gram creatinine have also been reported (196). Excretions greater than 0.5 µmol/L in 24-hour urine or 20 µg/g creatinine are considered indicative of vitamin B6 adequacy (110, 196).

Erythrocyte Pyridoxal 5′-Phosphate

Erythrocyte PLP has been suggested to be a more relevant marker of vitamin B6 status than plasma PLP because PLP serves as an intracellular cofactor (110, 239). It is positively correlated with vitamin B6 intake (76, 87), plasma PLP (213), PL (214, 237) and PA (76), urinary PA excretion (76), and erythrocyte aspartate transaminase (EAST) basal activity and its activation coefficient EAST-AC (81), and it responds within weeks to vitamin B6 depletion and repletion (76, 87). Erythrocyte PLP shows a massive, transient increase within one hour after ingestion of pyridoxine (100 mg) and then decreases rapidly over the subsequent four hours (180). Plasma PLP is less responsive (15, 96). Thus, erythrocyte PLP appears to be more responsive than plasma PLP to supplementation (176).

Compared to plasma PLP, erythrocyte PLP has other complementary characteristics. Most importantly, erythrocyte PLP is actually increased in critically ill patients with evidence of systemic inflammation and hypoalbuminemia and with a concurrent marked reduction in plasma PLP (214). In these patients, plasma and erythrocyte PLP are weakly correlated (214). Similarly, RA patients with chronic inflammation have normal erythrocyte PLP but low plasma PLP (36). Furthermore, ALP is an ectoenzyme with no or low activity inside the cells such as erythrocytes, which explains why erythrocyte PLP has no association (11, 76, 96, 181) or a positive association with the activity of ALP, attributed to increased uptake of PL by the erythrocytes (75, 138). ALP is not associated with erythrocyte PLP in hypophosphatasia, in familial hypophosphatemic rickets (181), in elderly people (96), or in pregnant women (11).

PLP binds with high affinity to hemoglobin, variants of which have different affinities, with sickle cell hemoglobin having a higher affinity for PLP than hemoglobin A (97). This explains why patients with sickle cell anemia have low plasma PLP in combination with markedly elevated erythrocyte PLP (158) and demonstrates how different hemoglobin variants may cause misleading results. The binding of PLP and PL by hemoglobin may partially buffer the rate and extent of decline of erythrocyte PLP in B6 deficiency.

In conclusion, erythrocyte PLP may be a more reliable marker than plasma PLP of vitamin B6 status under conditions and diseases associated with inflammation (213), altered ALP, and low albumin (237). Since erythrocyte PLP responds dramatically following supplementation, fasting blood samples should be used for measurement. However, erythrocyte PLP is affected by hemoglobin variants, and the assay is cumbersome, with variable recovery and low precision (180). In addition, data on reference values in different populations are sparse and somewhat inconsistent (96, 110, 213).

Plasma Vitamer Ratios and the PAr Index

The major circulating B6 vitamers—PLP, PL, and PA—show moderate to strong associations in plasma (12, 149), between plasma and erythrocytes (213, 237), and between plasma and CSF (2). The associations indicate that transport and metabolism of B6 vitamers are strictly regulated. Despite the fact that knowledge on regulation is incomplete, associations have been translated into assessment of ratios between vitamers to address specific aspects of B6 metabolism and distribution in healthy and diseased subjects. In general, ratios between closely related metabolites along a given pathway may attenuate the influence from confounding factors that affect the isolated metabolites. Here, we focus on the ratios between B6 vitamers in serum or plasma.

The plasma PLP:PL ratio is reduced by more than 50% in critically ill patients with elevated CRP, elevated ALP, and low serum albumin as compared with healthy controls. A similar but less pronounced reduction has been observed for the erythrocyte PLP:PL ratio. Plasma PLP:PL is inversely associated with serum albumin, suggesting that a decreased ratio may reflect low albumin in critically ill patients (237). Conceivably, the PLP:PL ratio fluctuates less in response to vitamin B6 intake than does plasma PLP viewed in isolation.

Plasma PA is increased in patients with renal failure and following vitamin B6 administration; the latter produces only small changes in the plasma PA:PL ratio. The PA:PL ratio shows a strong association with serum creatinine (0.72) and might become an auxiliary tool for the assessment of renal function (43).

The PA:PL, PA:PLP, and PA:(PLP + PL) ratios were validated as markers of vitamin B6 catabolism and compared in terms of within-person reproducibility (ICC) and determinants (229). PA:(PLP + PL), termed the PAr index, has some unique characteristics. It has a high ICC (0.75), and a set of four inflammatory markers accounts for more than 90% of the explained variance of PAr in a regression model adjusted for supplement intake, smoking, kidney function, age, and sex. In receiver operating characteristics (ROC) analysis, PAr efficiently discriminates high inflammatory status with an area under the curve (AUC) of 0.85. In comparison, individual B6 vitamers have only modest associations with inflammation and stronger associations with vitamin B6 and supplement intake, smoking, and kidney function (229). Thus, PAr stands out as a marker of the increased B6 catabolism that occurs during inflammation and has the potential to reveal processes involved in pathogenesis and predict the risk of inflammatory-related diseases (258).

B6 Vitamers in Cerebrospinal Fluid

Measurement of B6 vitamers in cerebrospinal fluid (CSF) is primarily motivated by a search for biomarkers for the diagnosis and follow-up in newborns with inborn errors of vitamin B6 metabolism, including pyridox(am)ine 5-phosphate oxidase (PNPO) deficiency (mutations in the *PNPO* gene; OMIM 610090), antiquitin deficiency (mutations in the *ALDH7A1* gene; OMIM 266100), hypophosphatasia (alkaline phosphatase deficiency; OMIM 241500), hyperprolinemia type II (pyrroline-5-carboxylate dehydrogenase deficiency; OMIM 239510), and molybdenum cofactor deficiency (173).

PL, PLP, and PA are present and positively correlated in CSF from newborns, with higher concentrations and higher PA:PL ratios found in preterm than older babies (236). In preterm babies, PL is the most abundant vitamer, and concentrations decrease in the order PL, PLP, and PA (3). In older children, CSF PLP decreased with age until about 2 years (63, 162). In adults, the CSF B6 vitamer composition (PL > PLP > PA) is similar to that found in children, and the concentration of each vitamer is strongly correlated with the respective vitamer in plasma (2). CSF PL is lower whereas PLP is higher in men than in women (2).

The composition and age-related changes of B6 vitamers in CSF from preterm babies and young children suggest immaturity of B6 homeostasis or may reflect a B6 requirement during central nervous system development. For the detection of moderate divergences in CSF biomarker status, additional studies are required to establish normal concentrations in age- and sex-related strata of healthy newborns, children, and adults and to identify possible effects from vitamin B6 supplementation and drug treatment (63). Existing knowledge on B6 vitamers in CSF may still be useful for the detection of a drastic reduction in B6 vitamers in CSF that prevails in some metabolic disorders (63) such as PNPO deficiency (162).

FUNCTIONAL VITAMIN B6 BIOMARKERS

The Transaminase Tests

Measurements of PLP-dependent transaminase activities have been extensively used for the assessment of vitamin B6 status. The enzymes are usually measured in packed erythrocyte extract, which has considerably more aspartic acid transaminase (AST; EC 2.6.1.1) than alanine transaminase (ALT; EC 2.6.1.2) activity (102). However, both erythrocyte AST (EAST) and erythrocyte ALT (EALT) respond to a change in vitamin B6 status and in vitro–supplied PLP (33, 210).

The specific tests used are the measurements of basal (endogenous) activities of these transaminases, denoted oEAST and oEALT, respectively, and the activities induced by the in vitro addition of PLP. The results from the latter measurement are given as an activation coefficient (AC), which is the activity with added PLP divided by the activity without added PLP, denoted EAST-AC (or alpha-EAST) and EALT-AC (or alpha-EALT), respectively. Thus, a higher AC reflects a lower vitamin B6 status. The measurement of such ratios overcomes some of the difficulties related to method differences and between-subject variability (157, 210).

EAST and EALT and their ACs are considered to be long-term indicators of vitamin B6 status related to the life span of the erythrocytes (110) and correlate with vitamin B6 intake (31). However, the correlations are weaker in some studies than for plasma PLP and PA excretion (24, 75). In two vitamin B6 depletion-repletion studies, endogenous EALT and its AC were more responsive than endogenous EAST and its AC to vitamin B6 intake (75, 102), but the transaminase tests require higher doses of vitamin B6 for normalization than does plasma PLP (75, 102). However, in another depletion-repletion study, both transaminase tests, plasma PLP, erythrocyte PLP, and urinary PA were, in effect, equally responsive to vitamin B6 intake (87). Notably, the transaminase tests are not related to albumin, ALP (24), immune indices (85), and kidney function, but the EAST-AC is weakly, inversely associated with the acute-phase reactant alpha1-antichymotrypsin in children and young adults (12).

Drawbacks of the transaminase tests are that fresh blood must be used within hours after acquisition (87) and that analysis in frozen erythrocyte samples will give false values for both endogenous activities and ACs. Furthermore, hemoglobin affects the transaminase activities, and the assays have been difficult to standardize. The transaminase apoenzymes increase in diseases associated with necrotic processes, and EAST decreases following alcohol intake (157, 210).

Plasma Kynurenines

The amino acid tryptophan is catabolized mainly through the kynurenine pathway, forming metabolites collectively referred to as kynurenines. The initial step in this pathway, catalyzed by indoleamine (2,3)-dioxygenase, is activated by proinflammatory cytokines (101, 107), and two downstream enzymes, kynurenine transaminase (KAT) and kynureninase (KYNU), require pyridoxal 5′-phosphate as cofactor. Details are given in **Figure 2**. Kynurenic acid (KA) and xanthurenic acid (XA) are both end-stage metabolites with high renal clearance (101, 107). Notably, KYNU activity is reduced during dietary vitamin B6 restriction and is more responsive than KAT to vitamin B6 deficiency (160, 234).

Due to the involvement of PLP as a cofactor in the kynurenine pathway, both plasma and urine contents of these metabolites have been evaluated as markers of vitamin B6 status. Recently, assays that determine wide panels of kynurenines have been published (145, 257), enabling large-scale population-based studies on kynurenines and health (219, 220).

Although all the kynurenines are stable in plasma for at least one year when stored at $-80°C$ (145), the stability of 3-hydroxykynurenine (HK) and to a larger extent 3-hydroxyanthranilic acid (HAA) decreases significantly in chilled EDTA and heparin blood (147), in serum and plasma at room temperature, and in serum at $-25°C$ (89). The within-person reproducibility of kynurenines over 1–3 years is good (ICC of 0.5–0.7) (147).

Among the kynurenines, only HK increases in plasma in vitamin B6–deficient subjects, which probably reflects that its removal but not formation involves PLP-dependent enzymes (**Figure 2**). The inverse association between plasma PLP and HK in plasma is nonlinear, with the strongest correlation observed below approximately 20 nmol/L PLP, which is often considered as a cut-off level for the detection of deficiency (110). Furthermore, plasma HK is decreased after vitamin B6 supplementation (148, 220). However, plasma HK also shows a strong relation with inflammatory markers (positive), kidney function (inverse), and circulating tryptophan (positive) (231), and the strong PLP-HK association is essentially confined to subjects with increased levels of inflammatory markers (148). These observations undermine the concept of plasma HK as a specific marker of vitamin B6 status.

Kynurenine metabolite ratios, including the substrate-product pairs HK/XA and HK/HAA, have been explored as markers of vitamin B6 status. Similarly to HK alone, both ratios show nonlinear, inverse associations with PLP, with a break point at approximately 20 nmol/L. However, HK/XA and HK/HAA discriminate low PLP better (AUC of 0.78 and 0.78, respectively, by ROC analyses) than does HK (AUC of 0.65) and decrease substantially after vitamin B6 supplementation (231). Compared with HK, the ratios HK:XA, HK:HAA, and HK:KA in particular show weaker or no association with inflammation, body mass index (BMI), and kidney function, which are strong determinants of most kynurenines (169, 219). These observations demonstrate the feasibility of using metabolite ratios as a strategy to cancel out the influence of potential confounders affecting the components (metabolites) in the numerator and denominator and point to metabolite ratios as useful markers of vitamin B6 status.

Plasma Amino Acids, Transsulfuration, and One-Carbon Metabolites

Vitamin B6 deficiency has been shown to alter the concentrations of metabolites related to one-carbon metabolism, some of which have been proposed as sensitive biomarkers of functional vitamin B6 deficiency. Plasma concentrations of glycine, cystathionine, serine, and creatine as well as the 2-oxoglutarate:glutamate ratio have been shown to change in response to vitamin B6 deficiency (49, 50, 73, 104, 159, 167).

In humans, plasma glycine concentration showed an increase of 28% after two weeks of vitamin B6 depletion with 0.16 mg/d pyridoxine, whereas serine showed an increase of 47% after only one week of depletion. Concentrations were decreased after pyridoxine supplementation (167). The increase in glycine and serine is in agreement with the 11–29% increase in plasma glycine and the 12% increase in plasma serine observed in healthy men and women after a 28-day dietary controlled vitamin B6 restriction that resulted in moderate vitamin B6 deficiency (49, 51, 104). Additionally, glycine concentration was significantly higher in HepG2 cells that were cultured in low vitamin B6 conditions compared to cells cultured in higher B6 concentrations (48). In rats, vitamin B6 deficiency results in increased glycine concentrations in liver (198), muscle, and plasma (212).

In one-carbon metabolism, glycine and serine are metabolized by the mitochondrial and cytoplasmic PLP-dependent serine hydroxymethyltransferase, and glycine is also metabolized via the glycine cleavage system by the PLP-dependent glycine decarboxylase (**Figure 4**). Increased glycine and serine concentrations may be the result from the reduced activity of these enzymes.
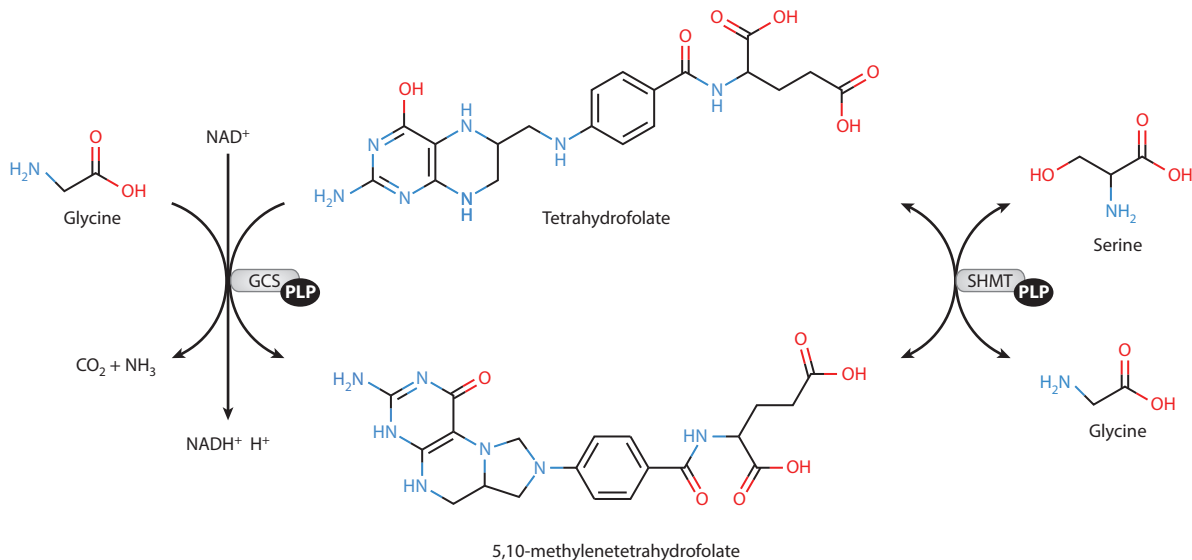
Figure 4

Serine hydroxymethyltransferase (SHMT) and the glycine cleavage system (GCS). SHMT (EC 2.2.2.1) is a vitamin B6 [pyridoxal 5′-phosphate (PLP)]–dependent enzyme that catalyzes the reversible conversion of serine to glycine. In mammals there are two isoforms, a cytoplasmic (cSHMT) and a mitochondrial (mSHMT) form. GCS is a mitochondrial multienzyme complex that is composed of four individual proteins, three specific components (P-, T-, and H-proteins), and one housekeeping enzyme, dihydrolipoamide dehydrogenase. P-protein is a vitamin B6 (PLP)–dependent glycine decarboxylase (glycine:lipoylprotein oxidoreductase; EC 1.4.4.2). This system catalyzes the oxidative cleavage of glycine.

However, although a decrease in serine hydroxymethyltransferase activity has been observed during deficiency (136), it is the reduction of the activity of glycine decarboxylase that appears to be the major cause of the accumulation of glycine and serine as demonstrated by mathematical modeling (104, 159). Accumulation of glycine can result in more glycine being converted to serine (159).

The concentration of cystathionine, a component of the transsulfuration pathway (**Figure 3**), is also elevated in inadequate vitamin B6 status (48, 50, 104). In humans, plasma cystathionine concentration increased by 53% in the preprandial state and 76% in the postprandial state in individuals with induced marginal deficiency (49). In vitamin B6–deficient rats, increased cystathionine was found in liver, muscle, and plasma (115, 212). Moreover, cystathionine was significantly higher in cells cultured in moderate vitamin B6 deficiency compared to cells cultured at more severe deficiency and normal B6 conditions (48). The observed increase in cystathionine in moderate vitamin B6 status can be explained by the different sensitivity of the enzymes cystathionine β-synthase and cystathionine γ-lyase to vitamin B6 concentration. Although moderate vitamin B6 deficiency results in decreased liver cystathionine γ-lyase activity, the activity of cystathionine β-synthase is maintained, causing the build-up of cystathionine (104, 115).

Plasma creatine was decreased after the 28-day dietary vitamin B6 restriction in healthy men and women (49). Additionally, creatine was significantly lower in cells cultured in low B6 conditions compared to cells cultured in intermediate or supraphysiological conditions (48). However, the decrease in creatine in human plasma and cells is in contrast to the increase found in the liver and muscle of vitamin B6-deficient rats (118). Thus the effects of vitamin B6 deficiency on creatine need further investigation.
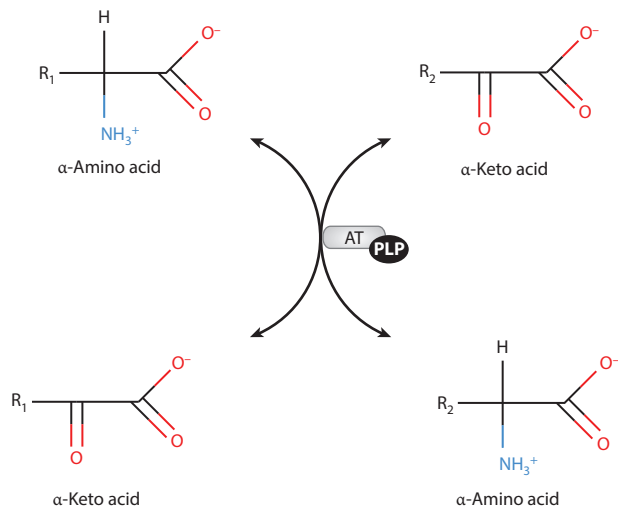
Figure 5

Aminotransferases (ATs). ATs catalyze the equilibration of amino groups among available alpha-keto acids. There are multiple ATs, and all require B6 [pyridoxal 5′-phosphate (PLP)] as a prosthetic group.

The significant increase of the 2-oxoglutarate:glutamate ratio (76%) observed after the 28-d dietary vitamin B6 restriction suggests that this ratio reflects an effect of vitamin B6 insufficiency reflecting functional alteration of this aminotransferase (**Figure 5**) (73).

Biomarkers in Urine

Measurements of urinary concentrations of vitamin B6, PA, cystathionine, and tryptophan catabolites including XA, Kyn, and HK have been used to evaluate vitamin B6 status (110). Because urinary excretion of PA and other B6 vitamers is considered to be a direct marker, it is discussed above.

Urinary cystathionine concentration in 24-hour urinary collections after an oral methionine load (3 g) has been used to evaluate vitamin B6 status (110, 166). Vitamin B6 deficiency results in an increase of urinary cystathionine concentration (110), which at levels >350 μmol/d may indicate deficiency (110). Pyridoxine supplementation corrects abnormal cystathionine excretion (166).

Increased XA in urine after a tryptophan load occurs in vitamin B6–deficient individuals (252). Tryptophan doses of 2 g and 5 g are most frequently used in adults, whereas a dose of 100 mg/kg body weight has been proposed as appropriate for children (46, 196). Assessment of XA excretion requires a 24-hour urine collection, and excretion of <65 μmol XA per 24 hours after a 2-g dose of tryptophan has been suggested to reflect adequate status (110). Urinary Kyn and HK have also been measured, but concentrations reflecting adequate vitamin B6 status are yet to be established. An increase in these metabolites compared to baseline concentrations has been observed in vitamin B6–deficient individuals (108), whereas a decrease in concentration occurs following pyridoxine supplementation (108). Urinary collections over shorter periods of time including 6 or 18 hours have been proposed as an alternative to the 24-hour urine collection (46, 125, 196). However, these short collection periods have been criticized due to the between-individual variability of metabolite excretion over time (196). In some individuals the excretion of the majority of the metabolites occurred during an 18-hour period (196). Tryptophan loads of 5 g increase the probability of finding abnormal metabolite concentrations in vitamin B6–deficient

individuals in the first 6-hour collection (196). For the 6-hour urinary excretion, an XA level of >25 mg was proposed as indicative of deficiency (196).

Although the tryptophan load test has been used for decades to determine vitamin B6 status, some limitations should be noted. Several factors and conditions have been reported to affect the concentration of the tryptophan metabolites in urine, including inflammation (163, 170), pregnancy (188), hormones (e.g., sex hormones, corticoids), and the use of certain drugs (e.g., oral contraceptives, theophylline, isoniazid, and deoxypyridoxine) (4, 188). Thus, the measurement of tryptophan metabolites for accurate assessment of nutritional vitamin B6 status in individuals with these conditions has been questioned. Loading tests in otherwise healthy individuals have shown to be useful and sensitive indicators of vitamin B6 status (196).

Mathematical modeling has provided information regarding the response of urinary tryptophan metabolites to different concentrations of vitamin B6 (183). The model that simulates tryptophan catabolism via the kynurenine pathway in the liver shows the response of urinary tryptophan metabolites in the absence and presence of tryptophan loads. In the absence of tryptophan loads, a slight increase in HK and a slight decrease in KA and AA are observed at moderate vitamin B6 deficiency. These changes become more pronounced as the deficiency becomes more severe. XA and Kyn increase only at a more pronounced deficiency, which suggests that HK, KA, and AA are more sensitive than XA and Kyn to vitamin B6 deficiency when the pathway is not stressed with tryptophan loading (183).

MULTIANALYTE APPROACHES

Advances in analytical methods allow more informative evaluation of vitamin B6 status by simultaneous evaluation of multiple biomarkers. This approach can provide information regarding various aspects of PLP-dependent metabolic processes that may differ in sensitivity to B6 deficiency or in rate of response to changes of B6 intake. Thus, the evaluation of multiple biomarkers can be more informative than the measurement of a single constituent. As described above, the measurement of the pattern of B6 vitamers can provide additional information to complement and extend the interpretation of a single vitamer such as PLP alone. Hence, the sum of B6 aldehydes and the B6 vitamer ratios merit further application in evaluating vitamin B6 status.

The measurement of patterns of specific chemical constituents of blood or urine through metabolite profiling or global metabolomic analysis identifies potential biomarkers and can give information about the substrates and products of PLP-dependent enzyme-catalyzed reactions and the overall fluxes of pathways containing PLP-dependent steps. Such analysis is now feasible in many settings including relatively small-scale nutritional intervention studies (49) and larger population-scale epidemiological studies (148, 231).

Targeted Analysis

The quantitative measurement of specific groups of chemicals is termed metabolite profile analysis or metabolic profiling, which is a subset of metabolomics (227). Contemporary methods for targeted analyses are based on mass spectrometry and inclusion of labeled internal standards, which enable quantification of low-abundance metabolites that often are tightly regulated and therefore may serve as responsive functional markers of vitamin B6 status. Such performance contrasts to that of untargeted, global analyses, which detect only abundant metabolites and with the inherent weakness of method interference.

Targeted analyses have been used for conventional measurement of, for instance, amino acids, fatty acids, organic acids, and acyl-carnitines in addition to mass spectrometry–based methods for

the determination of the constituents of one-carbon metabolism and related compounds (49, 146) and the tryptophan catabolites (145). For example, experimental dietary vitamin B6 restriction causes increased urinary and plasma glycine and cystathionine (49–51, 73, 104, 211), with similar results observed in larger studies (149). Vitamin B6 insufficiency is also associated with changes in the profiles of plasma organic acids (73) and plasma fatty acid profiles (256). The mechanism by which the patterns of n-3 and n-6 polyunsaturated fatty acids are altered in vitamin B6 insufficiency is unclear, and it is likely that fatty acid profiles will not serve as useful indicators of B6 status.

Measurement of the constituents of one-carbon metabolism and related pathways by LC-MS/MS (49, 146) provides a useful picture of the impact of low B6 status in analysis of plasma and parallel studies with cultured cells (48, 49). Such analysis provides information about glycine and cystathionine, which are potential functional biomarkers, but it also shows the more subtle influence on other constituents, including serine and dimethylglycine, and in more severe deficiency, total homocysteine. Indeed, total homocysteine is important because it is a sensitive biomarker of folate and vitamin B12 insufficiency and therefore aids interpretation if multiple deficiencies are present.

Functional identification of vitamin B6 insufficiency is strengthened by the concurrent targeted analyses of one-carbon and tryptophan metabolites. As discussed above, the measurement of a wide array of tryptophan catabolic products yields biomarkers of B6 insufficiency, such as the HK/XA, as well as other ratios that extend the diagnostic interpretation.

Global Metabolomic Analysis

In contrast to the specific quantitative analysis provided by the targeted methods described above, global metabolomic analysis evaluates all detected constituents of a sample such as plasma, urine, tissues, or cells and may therefore detect responsive metabolites that are not predefined (hypothesis generating). The most common global methods include nuclear magnetic resonance (NMR) and LC-MS/MS alone or with GC-MS or GC-MS/MS. NMR requires minimal sample preparation (54) and gives uniform response (based on H atoms) but has complex spectra and lower sensitivity than mass spectrometry. However, it is an excellent technique for detecting differences in the relatively high-abundance plasma or urine organic chemical constituents. Appropriate spectral analysis approaches allow estimation of concentrations. Because NMR has considerably lower sensitivity, it is well suited for research studies evaluating functional effects of vitamin B6 insufficiency that affect constituents such as amino acids and organic acids (73). In contrast, global LC-MS or LC-MS/MS procedures have the potential to detect several thousand plasma constituents (92), although less than half of them may be identified, and absolute quantification is generally not provided. Global metabolomic approaches will be useful in the discovery of potential new biomarkers, but such findings must be confirmed by targeted, quantitative methods. In addition to data reduction/visualization methods such as principal component analysis, partial least squares discriminant analysis, and orthogonal partial least squares discriminant analysis (222, 249), any form of targeted or global metabolomic analysis requires befitting statistical analysis, for example with multivariate analysis of variance or related approaches (37) for determination of significant discriminating biomarkers. Further discussion of these essential statistical considerations is beyond the scope of this review.

Diagnostic Possibilities from Multianalyte Analysis

The use of multiple biomarkers provides the option of diagnostic testing based on a profile of functional biomarkers. The high-throughput metabolite profiling methods currently available

provide such an opportunity and are presently under investigation by the authors. Although the routine use of NMR spectroscopy would be impractical for nutritional assessment, preliminary testing of an orthogonal partial least squares discriminant analysis model based on the patterns before and after vitamin B6 restriction (73) have proved effective when used in a predictive mode for classification based on individual spectra (J.F. Gregory, unpublished findings). Modeling analogous to the multianalyte diagnostic procedure proposed for vitamin B12 (59) and homeostasis model assessment as an index of insulin resistance (243) may also provide an integrated functional assessment tool for vitamin B6.

STANDARD REFERENCE MATERIALS

Comparisons of biomarker concentrations over time in a single laboratory and between laboratories require appropriate quality control protocols and reference materials to assure the reliability of data. In this regard, certified reference materials provide metrologically valid controls that are traceable in composition and methodology for use in validating laboratory performance and data reliability. Standard Reference Materials (SRMs) are such certified materials; they meet additional rigorous criteria defined by the US National Institute of Standards and Technology (NIST). For PLP, NIST has recently developed SRM 3950, Vitamin B6 in Frozen Human Serum (**https://www-s.nist.gov/srmors/view_detail.cfm?srm=3950**), which provides two pools of human serum with two certified concentrations for PLP and «information values» for PA. Two additional NIST SRMs are relevant to vitamin B6 biomarker assessment. SRM 1955, Homocysteine and Folate in Frozen Human Serum, provides a certified concentration of total homocysteine. SRM 1950, Metabolites in Human Plasma, has certified values for a wide range of constituents including 9 vitamins (including PLP), total homocysteine, and 17 other amino acids (with glycine, serine, and methione included). Unfortunately, we are not aware of certified reference materials for the other functional biomarkers discussed herein, including cystathionine, KYN, HK, HAA, and XA. Development of such reference materials would aid in the expanded assessment of these biomarkers for vitamin B-6 research and use in diagnostic procedures.

DEMOGRAPHICS AND LIFESTYLE

Pregnancy and Lactation

Increased excretion of kynurenines following tryptophan loading and low plasma PLP during pregnancy were reported decades ago (157, 200, 209). Erythrocyte aminotransferases and ACs are not affected by pregnancy and hormonal status (209). Notably, normalization of plasma PLP (200) and the tryptophan loading test (209) in pregnant women requires high doses of pyridoxine. Plasma PLP declines markedly during the second trimester, to approximately 30% of nonpregnant levels, and remains low between gestational week 30 and term before normalizing within weeks postpartum (81, 205, 221). During pregnancy there is a concurrent increase in PL, with essentially no change or a slight reduction in total B6 aldehyde (11, 221), an increase in erythrocyte PLP (67, 74), and no change in urinary PA excretion (221). The abnormal tryptophan loading test outcome may reflect hormonal effects on tryptophan catabolism (209), and reduced plasma PLP has been explained by dephosphorylation to PL catalyzed by ALP, which is increased during pregnancy (11). Low serum albumin in pregnant women coincides with low PLP, and the PLP:albumin ratio shows only a small reduction (11, 221). Normal PA excretion suggests adequate B6 intake and bioavailability in most pregnant women (205, 221), whereas a marked increase in PA clearance (43) can be attributed to expanded extracellular volume and increased glomerular filtration. Thus, changes in vitamin B6 biomarkers during pregnancy may not reflect common vitamin B6 deficiency

secondary to placental transport of vitamin B6 to the fetus, but rather a physiological response involving altered vitamin B6 distribution, including accumulation of PLP in erythrocytes, secondary to hormonal changes and hemodilution. Reference levels for the assessment of vitamin B6 status during pregnancy should be based on biomarker levels throughout pregnancy in healthy women.

Assessment of vitamin B6 status in lactating women is motivated by the observation that vitamin B6 content in breast milk is strongly associated with maternal plasma PLP (7, 32, 93, 156). Biomarker assessments of vitamin B6 status in lactating women have included plasma total vitamin B6, plasma PLP, erythrocyte PLP, erythrocyte transaminase activities (81), and urinary PA excretion and clearance (43). All of these indices have values in the ranges reported for healthy nonpregnant, nonlactating women, and all indices, except erythrocyte transaminase, respond to vitamin B6 intake and/or supplementation (43, 156).

Infancy

Plasma PLP in term infants decreases within days after birth as compared to the high PLP measured in cord blood. Yet infants still have up to 2–6 times higher plasma PLP levels than adults despite human milk-fed babies having elevated ALP (20, 21). After 6 months of age, plasma PLP declines to adult levels, independent of vitamin B6 intake (21).

Newborns also have higher erythrocyte PLP and aminotransferase activity and lower aminotransferase AC than their mothers. Like plasma PLP, a child's vitamin B6 status measured by erythrocyte PLP and aminotransferase tests declines during the first months of age (81, 93) and reaches adult levels at the age of about 5 years (80).

Vitamin B6 biomarkers are strongly correlated in infants (81). Vitamin B6 status in neonates is associated with vitamin B6 intake and supplementation of the mother and child (93) and is higher in formula-fed than in breast-milk-fed infants (94). The PL:PLP ratio, which correlates strongly with ALP, increases dramatically in neonates with low vitamin B6 intake (93), suggesting the formation of PL as a mechanism facilitating vitamin B6 transport in deficient infants. This emphasizes the utility of total B6-aldehyde as a vitamin B6 marker in infants.

The levels of plasma PLP, PL, erythrocyte, and whole-blood PLP and aminotransferase test and their relations are markedly different in premature as compared to term newborns. At birth, plasma PLP is similar in breast-fed preterm and term infants; however, PLP decreases within days in preterm infants to 11–40% of levels measured in term infants and is essentially nonresponsive to infant vitamin B6 supplementation (177) but varies in proportion to maternal vitamin B6 status. There is also a higher PL:PLP ratio in preterm infants than in term newborns (94), which may reflect increased ALP activity known to be elevated in preterm newborns (123). In contrast, erythrocyte and whole-blood PLP and blood total B6 are similar in preterm and term newborns and increase following supplementation of mother or infant or after formula feeding (94, 177).

The vitamin B6 biomarker profile in premature newborns has been explained by low B6 content in the milk of preterm mothers, interrupted fetal vitamin B6 accumulation, sequestration of PLP by erythrocytes, metabolic trapping of PLP by peripheral tissues or liver, reduced hepatic synthesis of PLP, and immaturity of vitamin B6–metabolizing enzymes (177). Whatever the mechanism, the available data demonstrate that in premature newborns plasma PLP is not a reliable marker of vitamin B6 status.

Age and Sex

Published data on age- and sex-related differences in vitamin B6 biomarkers are somewhat inconsistent, and many studies are based on a small number of subjects. After the high B6 status

in early infancy, erythrocyte PLP approaches adult levels at the age of 5 years (80). Erythrocyte PLP (80), plasma PLP (8, 12, 154), and plasma PA (8, 12) show no sex-related differences and are essentially stable between 1 and 11 years of age. After puberty, plasma PLP decreases as a function of age, at a rate of about 4 nmol/L per 10 year in nonsupplemented men (186). The decline is more pronounced in women, and in middle-aged women concentrations of plasma PLP are about 70% of that in middle-aged men (8, 154). In addition, plasma PL is higher in men than in women in this age group (55, 164). Plasma PA showed no (8) or minor (12) sex-related differences and is essentially stable up to the age of about 50 years. Above 60 years of age, plasma PLP continues to decline in men but increases in women, whereas plasma PA increases markedly in both sexes (8), leading to a higher plasma PA:PLP ratio in people aged 65 years and over (0.5–0.88) compared to younger people (0.22) (13). The age-related changes are attenuated in supplement users (154, 186).

The assessment of changes in vitamin B6 biomarker status related to increasing age and hormonal effects per se as opposed to dietary intake of vitamin B6 and protein, frailty, low-grade inflammation, and impaired renal function is challenging. Aminotransferase tests show no or inconsistent changes according to age, indicating that the functional or long-term consequences are questionable (24, 121, 164, 186).

Low plasma PLP in the elderly does not seem to be explained by low dietary vitamin B6 or low protein intake (13, 121), deficit in absorption, impaired synthesis, or retention of PLP in erythrocytes or liver (96). However, some (106) but not all (96, 164) authors suggest that the explanation is increased catabolism as reflected by increased urinary PA. The age-related decrease in albumin and, in particular, an increase in ALP will also lead to increased catabolism (12, 24, 96). The increase in plasma PA in both elderly men and women may be partly explained by impaired renal function (12).

Sex-related differences in ALP do not fully explain the postpubertal differences in PLP between the sexes since both PLP and PL are lower in women (55, 164). However, the increase in plasma PLP in postmenopausal women may partly reflect increased levels of inorganic phosphate inhibiting ALP (139).

Body Mass Index and Obesity

A positive correlation of vitamin B6 status (plasma PLP, PL, and 24-hour urine PA excretion) with body weight has been reported (24), whereas no association with BMI was found in two case-control studies comparing overweight and obese to normal-weight controls (78, 152). A high percentage of subjects undergoing gastric bypass surgery have low plasma PLP (22). The low serum PLP in 110 morbidly obese patients has been explained through markers that indicated low-grade chronic inflammation and PLP predictors such as ALP and inorganic phosphate (1).

Drugs

Certain drugs including hydralazine, penicillamine, isoniazid, phenelzine, cycloserine, thiamphenicol, and L-dopa may affect vitamin B6 status by covalent binding to the carbonyl groups of PLP and/or PL. Other drugs such as progabide, an antiepileptic drug, and theophylline may interfere with vitamin B6 metabolism by inhibiting pyridoxal kinase (53, 103).

Reduction in plasma PLP and PA in patients given antiepileptic drugs has been demonstrated in some (9, 10, 53) but not all (116) studies. A detailed investigation of impaired vitamin B6 status in patients treated with theophylline (226) demonstrated no change in plasma PL, but it did identify a rapid and drastic (50–70%) decline in plasma PLP followed by a decline in erythrocyte PLP and functional markers such as erythrocyte aminotransferase activities (224) as well as increased urinary excretion of XA after tryptophan loading (223). These effects are explained by theophylline being

a potent inhibitor of pyridoxal kinase (52, 103), which increases several-fold in erythrocytes during treatment, probably reflecting a compensatory mechanism to maintain adequate levels of PLP.

A recent study has demonstrated reduced plasma PLP but normal plasma PL in RA patients treated with conventional nonsteroidal anti-inflammatory drugs as well as selective cyclooxygenase-2 inhibitors. These drugs also change the concentrations of vitamin B6 forms in tissues of experimental animals (30). The mechanisms and functional implications of these observations are elusive, but the widespread use of these drugs makes them of potentially great importance.

Oral Contraceptives

A marked increase in urinary excretion of XA and other kynurenines after tryptophan loading in women using oral contraceptives (OCs) was reported almost 45 years ago (188). Increased excretion and its normalization with pyridoxine supplementation have since been confirmed in numerous studies (250), including studies on users of low-estrogen-dose OCs (131, 187). However, the tryptophan loading test is affected by factors independent of vitamin B6 status (110), including exogenous estrogen use (188), which may be explained by the effect of estrogens on KYNU and tryptophan (2,3)-dioxygenase activity (250). Findings on the associations of OC use with other vitamin B6 markers have been inconsistent (250). Reduced plasma PLP in women taking low-dose OCs was observed in the large-scale population-based National Health and Nutrition Examination Survey (2003–2004) (154), but the results from erythrocyte transaminase tests have been inconsistent. Studies on PA excretion show no difference between users and nonusers of OCs (250), and controlled depletion-repletion studies show normalization of direct and functional tests at the same pyridoxine dose in OC users and nonusers (109). Thus, the reduction of plasma PLP in OC users may reflect a shift from PLP to PL (140) and redistribution of PLP into tissues, possibly related to low-grade inflammation in OC users (250).

Alcohol

Moderate alcohol consumption is associated with improved vitamin B6 status (plasma PLP and EAST-AC) in several cross-sectional studies (57, 82, 122, 244). This has been attributed to the vitamin B6 content in beer. Notably, both vitamin B6 intake and plasma PLP increase in intervention studies examining beer consumption (14, 184, 235). Chronic alcoholism has consistently been associated with lowered plasma PLP (47, 68, 142), and a marked reduction in the serum alpha-aminobutyrate:cystathionine ratio in patients with alcoholic liver disease suggests impaired activity of the PLP-dependent cystathionine gamma-lyase and functional vitamin B6 deficiency in these patients (142). The association between low plasma PLP and alcohol consumption (24) may in part be explained by displacement of PLP from protein binding by acetaldehyde (128).

Smoking and Coffee Consumption

Smoking was associated with lower plasma PLP (202, 228, 245) but not with decreased erythrocyte PLP (239). Ex-smokers have plasma PLP levels that are intermediate to never smokers and current smokers (154, 239) and that approach levels of never smokers with increasing time (several years) since smoking cessation (228). Decreased plasma PLP could be caused by a smoking-associated increase in ALP (239), lowered albumin (203), altered tissue PLP distribution, and differences in dietary intake (228).

In a large cross-sectional study, consumption of coffee (≥4 cups/day) was associated with 14% lower plasma PLP (232). In several small intervention studies (with coffee and/or caffeine), no—or only a nonsignificant—decrease in plasma PLP has been observed (39, 233, 238).

Protein Intake

The requirement of vitamin B6 has been found to be dependent on protein intake in animals and humans (150, 151). Moreover, protein quality marginally affects vitamin B6 status in some (60) but not all studies involving rats (195) or humans (206). In most studies the ratio between vitamin B6 and protein intake was controlled by artificial diets, and inconsistent results may have been caused by severe amino acid imbalance (195). In human nutrition, food sources rich in protein (meat, liver, fish, beans, and lentils) are also among the best sources of vitamin B6 (see **http://www.nutrition.gov/smart-nutrition-101/dietary-reference-intakes-rdas**). Various direct vitamin B6 status indicators are positively associated with animal and plant protein intake, whereas functional indicators are not or only weakly associated to protein intake (25). Thus, rather than having a negative effect on vitamin B6 status, a protein-rich diet might moderately improve some measures of vitamin B6 status.

Vegetarianism

Vegetarians were reported to have similar (206) or lower (86, 88) plasma PLP than omnivores despite a similar (86, 206) or only marginally lower (88) vitamin B6 intake. The less-effective metabolic utilization of pyridoxine β-D-glucoside likely is responsible for any lower PLP levels associated with chronic consumption of plant-derived B6 sources (70). Dietary fiber associated with vegetarian diets was not found to adversely affect vitamin B6 bioavailability (69, 120, 206). In an Austrian study of omnivores, vegetarians, and vegans, 30% of the participants were vitamin B6 deficient according to EAST-AC, but vitamin B6 status (EAST-AC, plasma PLP, and PA excretion) was independent of diet form (132). An investigation of vegans showed a high percentage of marginal to low vitamin B6 status (EAST-AC) despite high vitamin B6 intakes (approx. 2.8 mg/day), and strict vegans were more affected than moderate vegans (242).

Exercise

A 24-week fitness-type exercise program did not appreciably affect vitamin B6 status (EAST-AC) in young women (61), nor were effects identified in a study on exercise-induced lactate in young adults (62) or in another study on moderate exercise among women (134). A transient increase in plasma PLP followed by a decrease after 1 hour was observed among three groups of women (young, young/untrained, postmenopausal/untrained) during the exercise period (134). Acutely elevated PLP may be metabolized to PA, as indicated by the consistent observations of increased PA excretion in active individuals or during exercise, suggesting increased loss or utilization of vitamin B6 (134). A fraction of athletes have lower functional vitamin B6 status (127), leading to the suggestion that exercise may increase the requirement for vitamin B6 (251).

Seasonal Variation

In 22 healthy adults from Northern Ireland, neither vitamin B6 intake nor status measured as EAST-AC showed seasonal variations (141). In Chinese women at childbearing age, plasma PLP was approximately 20% lower in winter and spring, and the prevalence of values <30 nmol/L decreased from >30% in spring to below 20% in fall (185). Seasonal variation in water-soluble vitamin status was also found in 61 pregnant women in Russia, with codeficiencies of vitamin B6 and carotenoids occurring both in spring and fall (133, 241). Thus, it appears that seasonal variations in B6 nutriture exist in areas where vitamin B6 intake reflects seasonal availability of specific foods and nutrients.

CONCLUSION

A variety of biomarkers of vitamin B6 status have been developed and validated during the past four decades. They are categorized into two main groups: direct biomarkers that measure B6 vitamers in plasma, blood, or urine; and functional biomarkers reflecting enzymatic or metabolic functions of vitamin B6. Key characteristics of these biomarkers are summarized in **Figure 6**.

Currently, direct biomarkers measured in plasma/serum, in particular plasma PLP, are most commonly used, mainly because of practicalities and increasing availability of contemporary methods that measure B6 vitamers with high sensitivity and high throughput. However, direct biomarkers based on PLP and PL, with the exception of erythrocyte PLP, are strongly influenced by inflammatory conditions and related factors such as increased ALP and low serum albumin, and
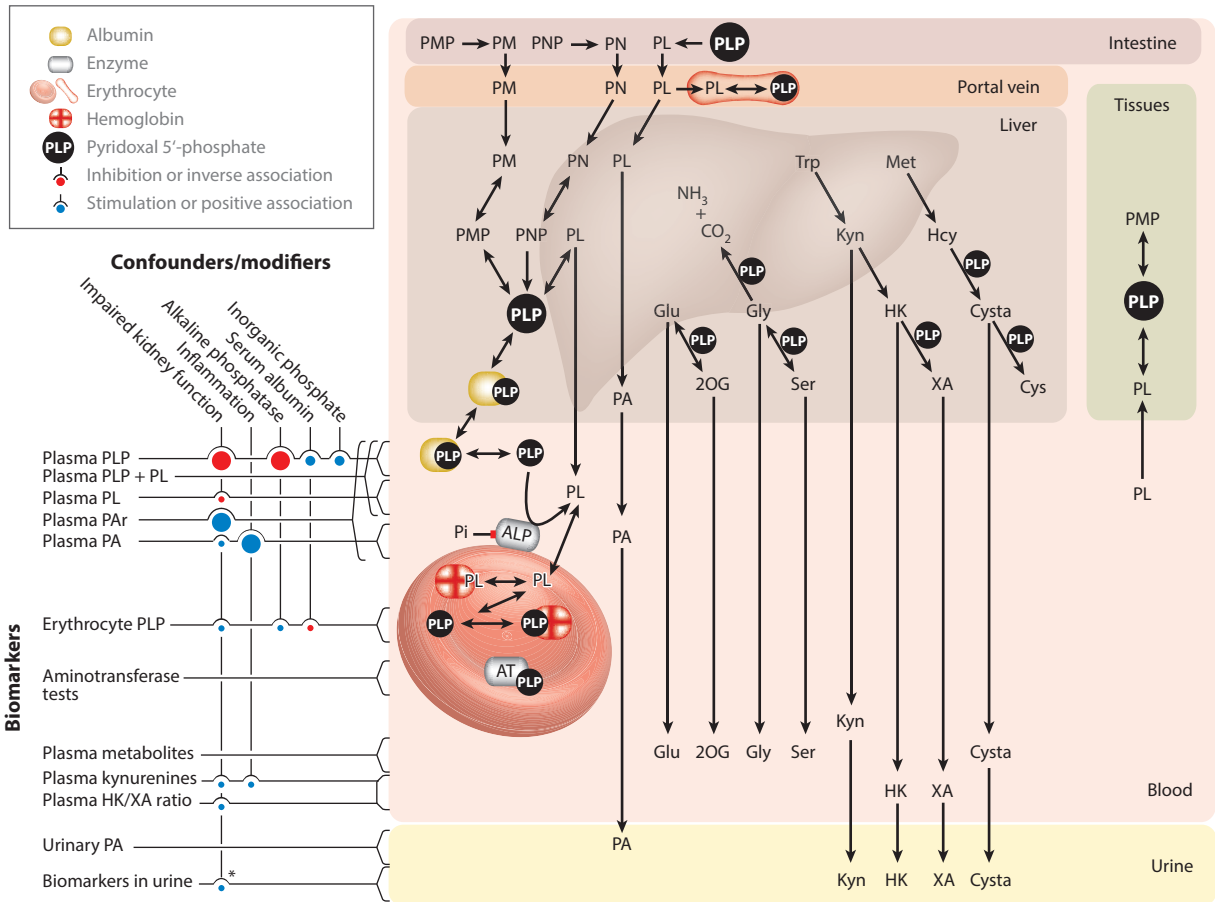

Figure 6

Main characteristics of vitamin B6 biomarkers in terms of primary confounders and linkage with metabolism and distribution of B6 vitamers and metabolites. The vertical lines at left with a bullet at the end indicate confounders with positive (*blue*) or negative (*red*) effect modification. The size of the bullets indicates the effect size. Abbreviations: 2OG, 2-oxoglutarate; AT, aminotransferase; Cys, cysteine; Cysta, cystathionine; Glu, glutamate; Gly, glycine; Hcy, homocysteine; HK, 3-hydroxykynurenine; Kyn, kynurenine; Met, methionine; PA, pyridoxic acid; PAr, PA:(PLP + PL) ratio; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine; PNP, pyridoxine 5′-phosphate; Ser, serine; Trp, tryptophan; XA, xanthurenic acid. *Urinary excretion of kynurenines may change during inflammation.

plasma PA increases with impaired renal function. These factors may confound the assessment of vitamin B6 status. Less confounding and increased specificity of direct biomarkers toward specified aspects of vitamin B6 metabolism have recently been documented by using ratios between B6 vitamers, as illustrated by the PAr index, which is a measure of vitamin B6 catabolism during inflammation.

Functional biomarkers based on erythrocyte aminotransferase activities may reflect long-term vitamin B6 status but currently are rarely used, mainly because of the requirement for fresh erythrocyte and owing to standardization difficulties. The tryptophan loading test based on urinary excretion of XA and other kynurenines is sensitive to B6 deficiency, but is an invasive and laborious procedure. Neither erythrocyte aminotransferase tests nor the tryptophan loading test is suitable for large-scale population-based studies, but they were appropriate tools for the assessment of vitamin B6 requirement. Recent vitamin B6 biomarker development has exploited analytical performance of liquid and gas chromatography coupled to mass spectrometry. These analytical developments have provided the opportunity to simultaneously quantify numerous amino acids and metabolites related to PLP-dependent pathways, including one-carbon metabolism, the transsulfuration pathway, and the kynurenine pathway. Ratios between substrate-product pairs or ratios between closely related metabolites have been considered as functional biomarkers. Whereas metabolites in isolation may be influenced by confounders such as inflammation and kidney function, the ratios are subject to less or no such influence, due to the positioning of metabolites as numerator and denominator. This strategy has provided promising candidate biomarkers, including the HK:XA ratio and, potentially, the 2-oxoglutarate:glutamate ratio. Future strategies involve the search for novel biomarkers using targeted metabolic profiling as well as untargeted metabolomic analysis, which may discover nonpredefined biomarkers. These efforts aim to discriminate individuals according to vitamin B6 status using a panel of metabolites and vitamers that are transformed into simplified derivatives using data-reduction statistics and summary scores.

SUMMARY POINTS

1. Direct vitamin B6 biomarkers measure B6 vitamers in plasma [pyridoxal 5′-phosphate (PLP), pyridoxal (PL), 4-pyridoxic acid (PA)], in erythrocyte (PLP), or in urine (PA).

2. Functional vitamin B6 biomarkers reflect the metabolic effects of PLP-dependent enzymes or pathways and include erythrocyte aspartic acid transaminase and its activation coefficient, plasma kynurenines, plasma amino acids (glycine and serine), transsulfuration (cystathionine), and one-carbon metabolites.

3. Some vitamin B6 biomarkers are influenced by factors not related to B6 status, such as inflammation (reduces plasma PLP), impaired kidney function (increases plasma PA), elevated alkaline phosphatase (reduces plasma PLP), low serum albumin (decreases plasma PLP), and elevated inorganic phosphate (increases plasma PLP).

4. Influence from some confounders listed under point 3 can be attenuated by using ratios between substrate-product pairs or ratios between adjacent metabolites, as demonstrated for the PAr index [PA:(PLP+PL)], which reflects increased vitamin B6 catabolism during inflammation, and the ratio between 3-hydroxykynurenine and xanthurenic acid (HK:XA), which is a functional marker of vitamin B6 status.

5. The within-subject reproducibility over time in terms of intraclass correlation coefficients has been determined for most but, regrettably, not all vitamin B6 markers.

6. Many demographic and lifestyle factors, including pregnancy, infancy, age, sex, BMI, some drugs, oral contraceptives, alcohol consumption, smoking, protein intake, vegetarianism, and exercise, may affect vitamin B6 biomarkers by mechanisms partly independent of vitamin B6 status, a fact that should be taken into account when establishing reference intervals and cut-off values.

FUTURE ISSUES

1. The growing recognition of conditions (inflammation, etc.) that influence plasma PLP concentration emphasizes the limitations of plasma PLP as a vitamin B6 biomarker in certain populations. Standardized methods are needed for the combined use of plasma PLP with other biomarkers for vitamin B6 status assessment.

2. Not all metabolic processes that require PLP exhibit the same sensitivity to vitamin B6 insufficiency. Additional information is needed regarding the responsiveness of the various functional biomarkers.

3. Development of functionally based cut-off values for vitamin B6 deficiency is a priority.

4. Development of additional reference materials for standardization of biomarker measurement is needed.

5. Further assessment of the usefulness of PLP + PL, the HK:XA ratio, and the 2-oxoglutarate:glutamate ratio as biomarkers is necessary.

6. The definition of the RDA for vitamin B6 on the basis of functional biomarkers should be considered.

7. Development of assessment tools based on the use of multiple functional biomarkers is needed.

DISCLOSURE STATEMENTS

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Errata

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