The metabolism of vitamin B6 in relation to genetic disease

Het metabolisme van vitamine B6 in relatie tot genetische ziekten

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CHAPTER 1

General Introduction

VITAMIN B6

Vitamin B6 is a water-soluble and for humans essential nutrient. It comprises six different vitamers, which all share the 2-methyl-3-hydroxypyridine structure but differ in the nature of the C4 and C5 substituents. The C4 substituents known for vitamin B6 are the hydroxymethyl group (-CH_2OH) in pyridoxine (PN), the aminomethyl group (-CH_2NH_2) in pyridoxamine (PM) and the aldehyde group (-CHO) in pyridoxal (PL). The C5 substituent for all these variants is either a hydroxymethyl group in the unphosphorylated B6 vitamers, or a hydroxymethyl group esterified to a phosphate in the phosphorylated forms of vitamin B6. [Clayton (2006)] (Figure 1)

Humans cannot synthesize vitamin B6 and must obtain it from sources in the diet. The different B6 vitamers are present in a wide variety of foods; in animal and milk products, mainly pyridoxamine phosphate (PMP) and pyridoxal phosphate (PLP) are present, whereas in plant-derived products, PN and pyridoxine phosphate (PNP) are the dominant B6 vitamers. Human breast milk primarily contains PL [Ooylan et al (2002)] and PN is widely used as a food supplement [Bender (2005)].

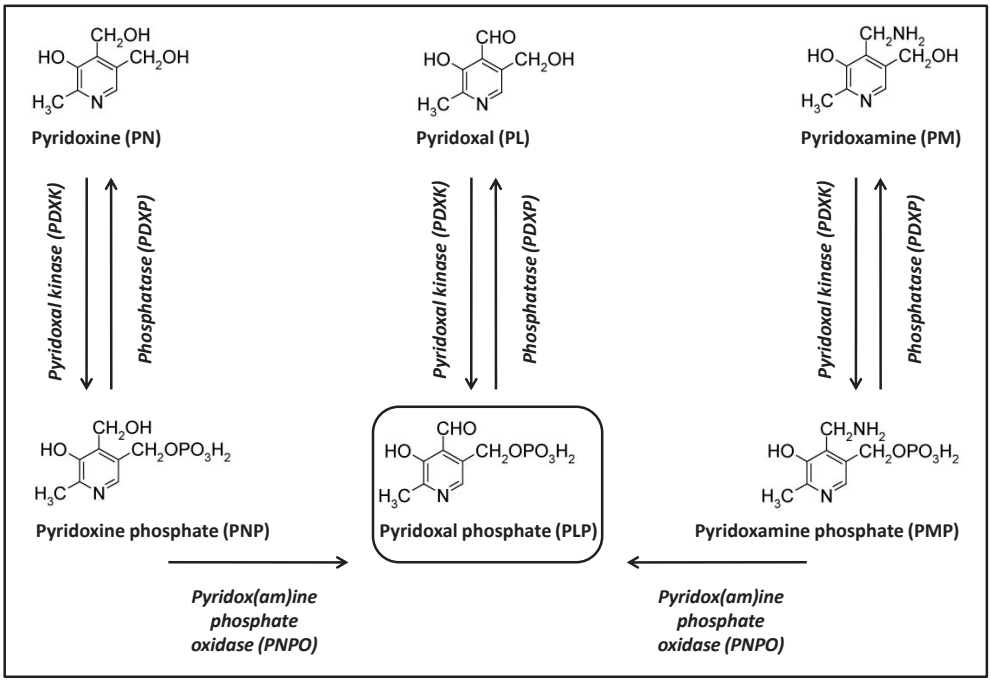

Figure 1 The different vitamin B6 vitamers and their intracellular conversions. Adapted from Albersen et al (2012).

Vitamin B6 metabolism and transport

Dietary B6 vitamers are absorbed in the small intestine by carrier-mediated transport. Transport across the cell membrane is preceded by hydrolysis of the phosphorylated B6 vitamers through the membrane-bound enzyme alkaline phosphatase (ALPL; EC3.1.3.1) [Spector and Greenwald (1978)] [Waymire et al (1995)]. *In vitro*, uptake of the unphosphorylated B6 vitamers by intestinal cells has been shown to be pH- and energy-dependent and to be under the regulation of extracellular substrate levels and an intracellular protein kinase A mediated pathway [Said et al (2003)] [Said et al (2008)].

Inside the cell, the unphosphorylated B6 vitamers are phosphorylated by pyridoxal kinase. PNP and PMP are then converted by pyridox(am)ine phosphate oxidase (PNPO) into PLP, the catalytically active form of vitamin B6 [Clayton (2006)]. Release of vitamin B6 from the cell is dependent on hydrolysis of the phosphorylated B6 vitamers by a vitamin B6-specific phosphatase [Jang et al (2003)]. Oxidation of PL by pyridoxal oxidase (EC1.2.3.8) [Merrill et al (1984)] constitutes the degradation pathway of vitamin B6, of which the major product, pyridoxic acid (PA), is excreted in urine [Bender (2005)].

Whereas the enzymes involved in vitamin B6 metabolism have been characterized at genetic and protein levels, literature is controversial regarding whether dietary vitamin B6 is metabolized in the intestine, or only taken up by intestinal cells and transported to the liver via the portal blood. Although the liver possesses the enzymatic system for the formation of PLP from the other B6 vitamers and although it is known that the liver metabolizes dietary vitamin B6 [Colombini and McCoy (1970)] [Contractor and Shane (1971)] [Johansson et al (1968)] [Johansson et al (1974)] [Lumeng et al (1974)] [Lumeng et al (1980)] [Mehansho et al (1980)] [Merrill et al (1984)], it has not been convincingly demonstrated that the liver is the main location of PLP formation. Furthermore, *in vivo* studies on vitamin B6 metabolism in mice suggest that dietary vitamin B6 is completely converted into PLP by the intestine and that the liver contributes to the formation of PLP only when the maximum capacity of the intestine to do so is exceeded [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)]. Until now, this discrepancy in literature has not been resolved.

The central nervous system is dependent on sources of vitamin B6 in blood, through transport across the blood brain barrier and choroid plexus. Knowledge on the mechanism(s) by which any of the unphosphorylated B6 vitamers is transported from blood to brain is limited and the relationship of B6 vitamer concentrations between blood and CSF is not known. At the biochemical level, there is evidence for carrier-mediated transport of vitamin B6 in blood brain barrier and choroid plexus [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies] [Spector and Johanson (2007)] but, similar to the situation in the

intestine and liver, a vitamin B6 transporter protein has not yet been characterized. Inside brain cells, PMP and PLP have been reported to be the dominant B6 vitamers [Sampson and O'Connor (1989)] [Sharma and Dakshinamurti (1992)]. Studies in rabbits have shown that brain cells only release the unphosphorylated B6 vitamers, whereas choroid plexus releases phosphorylated as well as unphosphorylated B6 vitamers into CSF [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies]. Concentrations of vitamin B6 were higher in rabbit brain than in rabbit plasma and CSF, suggesting active transport at the blood brain barrier and/or choroid plexus [Spector et al (2007)]. Despite the biochemical characterization of vitamin B6 uptake, vitamin B6 transporter proteins and their encoding genes have not yet been elucidated.

Pyridoxal kinase

Pyridoxal kinase (PDXK; EC2.7.1.35) catalyzes the phosphorylation of PL, PM and PN. This reaction is important, as it yields the phosphorylated B6 vitamers, which are poorly transportable across the cell membrane and thus are metabolically trapped inside the cell. Furthermore, phosphorylation provides the cell with PLP, either directly or through the PNPO-catalyzed conversion of PNP and PMP. The gene encoding pyridoxal kinase (*PDXK*; OMIM *179020) is located on chromosome 21q22.3. PDXK is a polypeptide of 312 amino acids probably acting as a dimer [Kerry et al (1986)] and its activity is dependent on ATP [Di Salvo et al (2004)].

Because only the unphosphorylated B6 vitamers can be transported across cellular membranes, there is a requirement for ubiquitous tissue expression of PDXK. Indeed, mRNA expression is found in numerous human tissues, with highest levels in testis, ileocaecum and pancreas [Kang et al (2004)]. The expression of PDXK in brain is 10-32% of that in testis. From *in vivo* rat studies, it is known that PDXK activity correlates well with regional concentrations of PLP. Brain areas with high PDXK activity have high concentrations of PLP and vice versa, so it is postulated that the activity of PDXK is coupled to the diversified systems requiring PLP [Ebadi et al (1978)].

Expression of PDXK is regulated by PARbZIP (proline and acidic amino acid-rich basic leucine zipper) transcription factors in liver and brain. PARbZIP proteins accumulate with circadian clock rhythms in peripheral tissues, but in most brain regions they are expressed at nearly invariable levels. [Gachon (2007)] PARbZIP protein deficient mice showed decreased levels of PLP, serotonin and dopamine and were highly susceptible to generalized epilepsies [Gachon et al (2004)]. This suggests that the expression of PDXK has to remain within narrow limits in the brain. *In vivo* studies in rats have demonstrated that vitamin B6 deprivation

leads to differential reduction of PDXK activity in tissues, with a significantly higher reduction in liver than in brain. The relatively small reduction of PDXK activity in brain may reflect a mechanism to preserve the vitamin B6 content of brain [Meisler and Thanassi 1980].

Pyridox(am)ine phosphate oxidase

Pyridox(am)ine phosphate oxidase (PNPO; EC1.4.3.5) is the enzyme catalyzing the formation of PLP from two different substrates, PNP and PMP. FMN (flavin mononucleotide) is the indispensable cofactor and oxygen functions as electron acceptor. The capacity of PNPO to oxidize both a primary amine and a primary alcohol by different reaction mechanisms, in order to form the same product, is unique among flavoprotein oxidases. In addition to its role in PLP synthesis, PNPO may have a function in the recycling of PMP, a product of transamination and decarboxylation reactions. The gene encoding pyridox(am)ine phosphate oxidase (*PNPO*; OMIM *603287), a 261 amino acid protein, is located on chromosome 17q21.32. Two identical subunits form a dimer, which binds two molecules of the cofactor FMN.

PNPO mRNA expression is detectable is various human tissues, with the highest levels in liver, kidney and skeletal muscle [Kang et al (2004)]. The expression of PNPO in brain is up to 26% of that in liver and is developmentally regulated: in fetal brain, PNPO expression is 12.5-fold lower than in adult brain [Kang et al (2004)]. Furthermore, PNPO is inhibited by PLP, a regulation mechanism with physiological importance in both liver and brain tissues. PLP probably exerts its regulatory role by binding to a specific lysyl residue in the dimer protein [Choi et al (1987)]. The tissue-specific reduction of enzyme activity in vitamin B6-deprived rats, as was observed for PDXK [Meisler and Thanassi 1980], does not apply to PNPO, since there was no effect of vitamin B6 deprivation on PNPO activity in rat liver and brain [Meisler and Thanassi 1980].

Vitamin B6-specific phosphatase

The vitamin B6-specific phosphatase enzyme (pyridoxal phosphatase or PDXP; EC3.1.3.74) catalyzes the intracellular hydrolysis of the phosphorylated B6 vitamers. The *PDXP* gene (OMIM *609246) is located on chromosome 22q12.3 and encodes a 296 amino acid protein. PDXP is, like PDXK and PNPO, differentially expressed in human tissues, with the highest levels in cerebral cortex, liver and testis [Kang et al (2004)]. The expression of PDXP in other brain regions is 38-94% compared to cerebral cortex. The relatively high expression of PDXP in brain may indicate that this enzyme has a specific role in the central nervous system. The

first step in the degradation of vitamin B6, hydrolysis of PLP into PL, is dependent on the action of PDXP [Jang et al (2003)].

Functions of vitamin B6

PLP is well known for its cofactor function in numerous enzymatic reactions in the central nervous system, where it mainly catalyzes amino acid and neurotransmitter metabolism. PLP is important for the biosynthesis of dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), glycine and D-serine [Fuchs et al (2005)], which suggests an essential role for vitamin B6 in normal brain development and functioning. In addition, PLP is required for the actions of, amongst >160 other enzymes [Percudani et al (2009)], glycogen phosphorylase (glucose biosynthesis), cystathionine β-synthase (homocysteine metabolism) and aminolevulinate synthase (heme biosynthesis). PLP-dependent enzymes also play an important role in the synthesis of neuroprotective compounds in the brain, such as kynurenic acid, an intermediate in the degradation pathway of tryptophan, which is formed by kynurenine aminotransferase [Han et al (2008)]. Kynurenic acid is an antagonist of the NMDA (N-methyl-D-aspartate) subtype of glutamate receptors and of the alpha-7-nicotinic acetylcholine receptor.

Inverse relationships of vitamin B6 with oxidative stress [Chetyrkin et al (2011)] [Keles et al (2010)] [Mooney et al (2009)], inflammation [Lotto et al (2012)] [Morris et al (2010)] [Paul et al (2013)] [Sakakeeny et al (2012)] [Ulvik et al (2012)], cardiovascular disease [Dhalla et al (2013)], diabetes [Kiran et al (2011)] and cancer [Banque et al (2012)] [Chou et al (2011)] [Galluzzi et al (2012)] [Galluzzi et al (2013)] [Harris et al (2012)] [Johansson et al (2010)] [Larsson et al (2010)] [Le Marchand et al (2011)] [Zhang et al (2011)] [Zschäbitz et al (2013)] have been reported. Regarding the latter, higher vitamin B6 intake was associated with a lower risk of colorectal cancer [Banque et al (2012)] [Larsson et al (2010)] [Zschäbitz et al (2013)] as well as a lower risk of breast cancer [Chou et al (2011)] [Zhang et al (2011)]. Higher concentrations of serum PLP were associated with a lower risk of lung cancer [Johansson et al (2010)]. In contrast, lower concentrations of plasma PLP have been associated with poorer cognition [Moorthy et al (2012)] [Riggs et al (1996)].

Vitamin B6 deficiency

An acquired deficiency of vitamin B6 due to malnutrition is rare, since the different B6 vitamers are abundantly present in food products and the bioavailability is around 75% [Bowling et al (2011)]. Malabsorption of B6 vitamers from the diet, endogenous and exogenous nucleophiles (which inactivate PLP) or drugs which influence the enzymes

involved in vitamin B6 metabolism may also lead to functional vitamin B6 deficiency [Clayton (2006)] [Footitt et al (2011)].

Clinical consequences of vitamin B6 deficiency are serious and mainly include neurological symptoms, of which seizures are the most distinctive. In addition, low levels of plasma PLP have been associated with depression [Hvas et al (2004)] and Alzheimer's disease [Miller et al (2002)] [Mulder et al (2005)] [Mulder et al (2007)]. Over the past years, interest in vitamin B6 has increased, since specific inborn errors of metabolism resulting in functional vitamin B6 deficiency, which will be discussed in the following paragraphs, have been identified. Patients present with epilepsy and, frequently, developmental delay [Stockler et al (2011)]. Several autosomal recessive disorders are known to cause functional vitamin B6 deficiency.

PNPO deficiency (pyridoxal phosphate dependent epilepsy)

Deficiency of PNPO (OMIM #610090) [Mills et al (2005)] leads to severe neonatal seizures (neonatal epileptic encephalopathy). In general, these seizures do not respond to conventional anti-epileptic drugs and can only be terminated by PLP administration (hence the name PLP-dependent epilepsy). Worldwide, only 18 patients have been reported [Veerapandiyan et al (2011)], which likely is an underestimated number. Intra-uterine seizures, premature birth with asphyxia, hypoglycemia and lactic acidosis may accompany the clinical picture. If left untreated, PNPO deficiency can be fatal, whereas early supplementation with PLP may restrict the usually observed developmental delay.

PNPO deficiency is caused by mutations in the *PNPO* gene, for which patients are homozygous or compound heterozygous [Hoffmann et al (2007)] [Khayat et al (2008)] [Mills et al (2005)] [Ruiz et al (2008)]. As a consequence, intracellular PNP and PMP cannot be converted into PLP, rendering the cell dependent on PL as the only precursor B6 vitamer for the formation of PLP. The resulting systemic PLP deficiency may explain the broad range of organ involvement that can be present in PNPO deficient patients. Apparently, dietary sources, including breast milk as well as placental transfer of PL, are inadequate for sufficient supply of PL for the formation of PLP, since seizures can already occur before birth.

Antiquitin deficiency (pyridoxine-dependent epilepsy)

The etiology of pyridoxine-dependent epilepsy (antiquitin deficiency; OMIM #266100) [Mills et al (2006)] differs significantly from that of PNPO deficiency, since PLP is inactivated by an accumulating intermediate of hampered lysine degradation. The estimated incidence of antiquitin deficiency in The Netherlands is 1:276000 newborn infants [Bok et al (2007)].

Patients carry a mutation in the *ALDH7A1* gene located on chromosome 5q31 and encoding the antiquitin enzyme (α-AASA (aminoadipic semialdehyde) / P6C (piperideine-6-carboxylate) dehydrogenase), which plays a central role in the brain-specific lysine degradation pathway. Antiquitin catalyzes the breakdown of α-AASA as well as P6C, the cyclic form of α-AASA being in equilibrium with α-AASA, into α-aminoadipate. Without proper functioning of antiquitin, α-AASA and P6C accumulate and the latter scavenges PLP by a Knoevenagel condensation reaction [Mills et al (2006)].

Numerous autosomal recessive mutations in *ALDH7A1* have been reported to date, but without a clear genotype-phenotype relation [Mills et al (2010)]. The onset of seizures is usually shortly after birth, but atypical presentations can occur even up to three years of life. Like PNPO deficiency, antiquitin deficiency in general is refractory to common anti-epileptic drugs, although some patients show partial responsiveness. Antiquitin deficiency can be treated with either PN or PLP. Discontinuation of treatment results in seizure recurrence. Antenatal treatment with PN has been reported to be possibly beneficial [Bok et al (2010)] and dietary lysine restriction is currently under investigation as an additional treatment option [Van Karnebeek et al (2012)].

Other vitamin B6 deficiencies

Other inherited metabolic disorders causing functional vitamin B6 deficiency are congenital hypophosphatasia (alkaline phosphatase (ALPL) deficiency; OMIM #241500) [Waymire et al (1995)] [Whyte et al (1988)] and hyperprolinaemia type II (pyrroline-5-carboxylate (P5C) dehydrogenase deficiency; OMIM #239510) [Walker et al (2000)]. In congenital hypophosphatasia, impaired dephosphorylation of extracellular PLP leads to impaired uptake of PL by the cell and a reduction of intracellular PLP, while levels of PLP in plasma are elevated. The resultant seizures can be treated with PN [Nunes et al (2002)]. Recently, normalization of plasma PLP levels in these patients has been reported upon ALPL-replacement therapy [Whyte et al (2012)]. In hyperprolinaemia type II, deficiency of the P5C dehydrogenase enzyme, which plays a role in proline degradation, leads to accumulation of P5C and scavenging of PLP by a Knoevenagel condensation reaction, like in antiquitin deficiency. In addition to these defects, there are genetically yet uncharacterized conditions that are responsive to vitamin B6 [Kanno et al (2007)] [Schmitt et al (2010)] [Veerapandiyan et al (2011)].

The use of PN or PLP to treat the abovementioned functional vitamin B6 deficiencies, including PNPO and antiquitin deficiency, is in some cases quite successful but in others only partly effective. A substantial number of affected children still suffers from developmental

delay and neurological symptoms, despite early treatment with large doses [Bok et al (2012)] [Stockler et al (2011)].

Diagnosing vitamin B6 deficiency

B6 vitamers in human plasma and CSF

In plasma, B6 vitamer concentrations have been determined using different high performance liquid chromatography (HPLC) based methods [Bates et al (1999) – Clin Chim Acta] [Bates et al (1999) – Public Health Nutrition] [Driskell et al (2000)] [Footitt et al (2013)] [Marszałł et al (2009)] [Midttun et al (2007)] [Midttun et al (2009)] [Sassi et al (2002)] [Shephard et al (1987)] [Talwar et al (2003)]. The B6 vitamers present in human plasma are PLP and PL. The degradation product of vitamin B6, pyridoxic acid, is also found in plasma. In cerebrospinal fluid (CSF), methods for quantification of vitamin B6 are based on HPLC with fluorescence detection [Footitt et al (2011)] [Ormazabal et al (2008)] or radioactive tyrosine decarboxylase assays [Shin et al (1984)]. With these methods, only PLP has been studied whereas concentrations of PL, PM(P), PN(P) and PA in CSF have not yet been described.
Decreased or elevated B6 vitamer concentrations may be used for diagnosing known functional vitamin B6 deficiencies as well as yet uncharacterized disorders of vitamin B6 metabolism and/or transport. Decreased concentrations of PLP [Goyal et al (2013)] [Mills et al (2005)] [Ruiz et al (2008)] and PL [Mills et al (2005)] have been found in CSF of patients with PNPO deficiency as well as antiquitin deficiency (decreased PLP only [Footitt et al (2011)]). Concentrations of the other B6 vitamers were not reported, while these might be abnormal as well and have differential diagnostic impact. In plasma, B6 vitamer concentrations have been reported only in PNPO- and antiquitin-deficient patients receiving vitamin B6 supplementation [Footitt et al (2013)].

Secondary biochemical abnormalities

In plasma and CSF of patients affected with PNPO and antiquitin deficiencies, changes in amino acid and neurotransmitter metabolite profiles have been found, probably as a consequence of impaired functioning of PLP-dependent enzymes involved in amino acid and neurotransmitter metabolism.

In CSF of PNPO-deficient patients, concentrations of threonine, 3-methoxytyrosine (L-dopamine metabolite) and glycine are elevated, whereas concentrations of the dopamine metabolite homovanillic acid (HVA) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) are decreased [Mills et al (2005)]. In plasma, elevated concentrations of

threonine as well as decreased concentrations of serotonin may be found. In urine of PNPO-deficient patients, concentrations of vanillactic acid can be increased [Khayat et al (2008)] [Mills et al (2005)] [Ruiz et al (2008)]. In patients with antiquitin deficiency, concentrations of pipecolic acid [Plecko et al (2005)] and α-aminoadipic semialdehyde (α-AASA) [Mills et al (2006)], the substrate of antiquitin, are increased in urine, plasma and CSF. Furthermore, concentrations of threonine and 3-methoxytyrosine may be elevated in CSF and increased concentrations of glycine have been found in plasma as well as CSF [Mills et al (2010)].

However, biochemical abnormalities are not always present and therefore, diagnosing can be difficult [Hoffmann et al (2007)] [Khayat et al (2008)] [Mercimek-Mahmutoglu et al (2013)] [Mills et al (2005)]. In other words, disturbances of vitamin B6 metabolism may be missed by biochemical profiling of the secondary effects of functional vitamin B6 deficiency. Direct analysis of B6 vitamers may overcome this diagnostic limitation and in general will increase our insight in normal human vitamin B6 metabolism and transport as well as B6 vitamer concentrations in health and disease.

We do not know the physiological importance of each B6 vitamer, nor the best way to diagnose and treat functional vitamin B6 deficiency. Although B6 vitamer concentrations have been reported for plasma and there is limited knowledge on PLP concentrations in CSF, the B6 vitamer distribution within brain cells and the intracellular effects of functional vitamin B6 deficiency as well as vitamin B6 supplementation are not known. In literature, ingestion of large doses of PN has been reported to be toxic [Hartmann et al (2011)] [Jortner (2000)] and to result in polyneuropathy [Jortner (2000)]. The mechanism of this neurotoxicity has not yet been elucidated.

OUTLINE OF THE THESIS

Over the past years, interest in vitamin B6 has increased, since its essential role in normal brain development and functioning has been recognized and specific inborn errors of metabolism resulting in functional vitamin B6 deficiency have been identified. Patients suffering from PNPO and antiquitin deficiencies are severely neurologically affected and despite early treatment with PLP and PN, their developmental outcome is often suboptimal. These disorders, as well as other disturbances of vitamin B6 metabolism and/or transport, are probably missed regularly, because secondary biochemical abnormalities are not always present and there are yet uncharacterized causes of functional vitamin B6 deficiency. In addition, decreased concentrations of PLP have been only scarcely reported in CSF of PNPO and antiquitin deficient patients and no information is available regarding concentrations of the other B6 vitamers in CSF.

In this study, we aimed to obtain insight in B6 vitamer concentrations in plasma and CSF of healthy subjects **(Chapter 2, 3 and 4)**, to deepen our understanding of the genetic regulation of B6 vitamers **(Chapter 5)**, to investigate the role of the intestine in human vitamin B6 metabolism **(Chapter 6)** and to study uptake, conversion and excretion of B6 vitamers by neuronal cells **(Chapter 7)**.

The first aim of this study was to obtain insight in B6 vitamer concentrations in plasma and CSF of healthy subjects in order to further elucidate human vitamin B6 metabolism and transport. We hypothesized that with direct analysis of vitamin B6 in body fluids, functional vitamin B6 deficiency can be reliably diagnosed and the biochemical effects of supplementation with PLP and PN can be monitored. This will contribute to overcoming the limitations currently hampering optimal diagnosis and treatment of disorders underlying functional vitamin B6 deficiency.

Although in human plasma, B6 vitamer concentrations have been reported, knowledge on B6 vitamer concentrations in human CSF is limited. We therefore developed and validated a rapid, sensitive and accurate UPLC-MS/MS (ultra performance liquid chromatography - tandem mass spectrometry) method for the simultaneous quantification of PL(P), PM(P), PN and PA in human CSF **(Chapter 2)**. We studied the presence of a rostrocaudal B6 vitamer concentration gradient, as well as the influence of exposure to light and storage temperature on CSF B6 vitamer concentrations. We used our UPLC-MS/MS method to quantify B6 vitamers in CSF obtained from 36 newborn infants **(Chapter 3)**, 90 children and >500 adults **(Chapter 2 and 4)** as well as seven children with PN or PLP supplementation **(Chapter 2 and 7)**. We additionally investigated the effects of cells and protein in CSF, sex, age, prematurity, type of nutrition, epilepsy and anti-epileptic drug treatment.

Furthermore, we adapted our UPLC-MS/MS method to enable the analysis of plasma and studied B6 vitamer concentrations in plasma obtained from a subgroup of the abovementioned subjects in which both body fluids were drawn simultaneously (70 children and >500 adults) **(Chapter 4)**. In this way, we were able to directly compare the B6 vitamer composition of both body fluids and draw conclusions regarding the relationships for the different B6 vitamers *in* as well as *between* plasma and CSF. Eventually, disturbances in these relationships may be used for identification of possible deficiencies of the enzymes involved in vitamin B6 metabolism or may point towards a problem in vitamin B6 transport.

The B6 vitamer composition of plasma and CSF is possibly influenced by dietary intake, metabolism and/or transport, as well as yet undefined, genetic factors. Although the enzymes involved in vitamin B6 metabolism have been elucidated at genetic and protein

levels, knowledge on vitamin B6 transport is limited. At the biochemical level, there is evidence for carrier-mediated transport in the intestine as well as choroid plexus and blood brain barrier, but a vitamin B6 transporter protein has not yet been characterized. To deepen our understanding of the genetic regulation of B6 vitamers, we conducted a GWAS (genome-wide association study) of B6 vitamer concentrations and ratios in and between plasma and CSF of almost 400 healthy adults and studied the association with common genetic variants **(Chapter 5)**.

Vitamin B6 is the term used to indicate a group of unphosphorylated and phosphorylated pyridine compounds that can be enzymatically interconverted. These interconversions are essential, since dietary sources mostly contain PN(P) and PM(P) whereas the biologically active cofactor of vitamin B6 is PLP. The organs that are important in the conversion of precursor B6 vitamers into PLP however, have not been irrefutably identified. Although it has been generally accepted to be the liver, there is a discrepancy in literature regarding the main location of vitamin B6 metabolism, because the intestine has also been implicated. We investigated the role of the intestine in human vitamin B6 metabolism using an *in vitro* model for intestinal enterocytes and studied uptake, metabolism and excretion of PN, PM and PL as well as expression of the enzymes involved in intracellular vitamin B6 metabolism (PDXK, PNPO and PDXP) **(Chapter 6)**.

Neither the normal B6 vitamer distribution within brain cells nor the intracellular consequences of functional vitamin B6 deficiency and vitamin B6 supplementation are known, while PN-related neurotoxicity has been reported in literature. In order to optimize diagnosis, treatment and possibly also outcome of PNPO deficiency, we investigated uptake, metabolism and excretion of PN, PM and PL in an *in vitro* model for neuronal cells and studied the effects of PNPO deficiency and supplementation with PN and PLP **(Chapter 7)**.

In **Chapter 8**, our findings are discussed and our perspectives are outlined.

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CHAPTER 2

Quantification of vitamin B6 vitamers in human cerebrospinal fluid by ultra performance liquid chromatography - tandem mass spectrometry

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ABSTRACT

Since vitamin B6 is essential for normal functioning of the central nervous system, there is growing need for sensitive analysis of B6 vitamers in cerebrospinal fluid (CSF). This manuscript describes the development and validation of a rapid, sensitive and accurate method for quantification of the vitamin B6 vitamers pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), pyridoxic acid (PA), pyridoxal 5'-phosphate (PLP), pyridoxamine 5'-phosphate (PMP) and pyridoxine 5'-phosphate (PNP) in human CSF.

The method is based on ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) with a simple sample preparation procedure of protein precipitation using 50 g/L trichloroacetic acid containing stable isotope labeled internal standards: PL-D_3 for PL and PM, PN-$^{13}C_4$ for PN, PA-D_2 for PA and PLP-D_3 for the phosphorylated vitamers. B6 vitamers were separated (Acquity HSS-T3 UPLC column) with a buffer containing acetic acid, heptafluorobutyric acid and acetonitrile. Positive electrospray ionization was used to monitor transitions *m/z* 168.1 → 150.1 (PL), 169.1 → 134.1 (PM), 170.1 → 134.1 (PN), 184.1 → 148.1 (PA), 248.1 → 150.1 (PLP), 249.1 → 232.1 (PMP) and 250.1 → 134.1 (PNP).

The method was validated at three concentration levels for each B6 vitamer in CSF. Recoveries of the internal standards were between 93% and 96%. Intra- and inter-assay variations were below 20%. Accuracy tests showed deviations from 3% (PN) to 39% (PMP). Limits of quantification were in the range of 0.03 nM to 5.37 nM. Poor results were obtained for quantification of PNP.

The method was applied to CSF samples of 20 subjects and two patients on pyridoxine supplementation. Using minimal CSF volumes this method is suitable for implementation in a routine diagnostic setting.

INTRODUCTION

Vitamin B6 is a water-soluble and for humans essential nutrient. It comprises different vitamers: the alcohol pyridoxine (PN), the aldehyde pyridoxal (PL), the amine pyridoxamine (PM), their phosphate esterified forms and pyridoxic acid (PA). Vitamin B6 metabolism includes several steps involving various enzymes [Clayton (2006)], starting with carrier mediated uptake of the B6 vitamers from dietary resources in the small intestine [Said et al (2003)].

Prior to transport across the cell membrane, phosphorylated B6 vitamers must be hydrolysed by a membrane-bound alkaline phosphatase. Intracellular (re-)phosphorylation by pyridoxal kinase is followed by a pyridox(am)ine phosphate oxidase (PNPO) mediated conversion of pyridoxine phosphate (PNP) and pyridoxamine phosphate (PMP) into pyridoxal

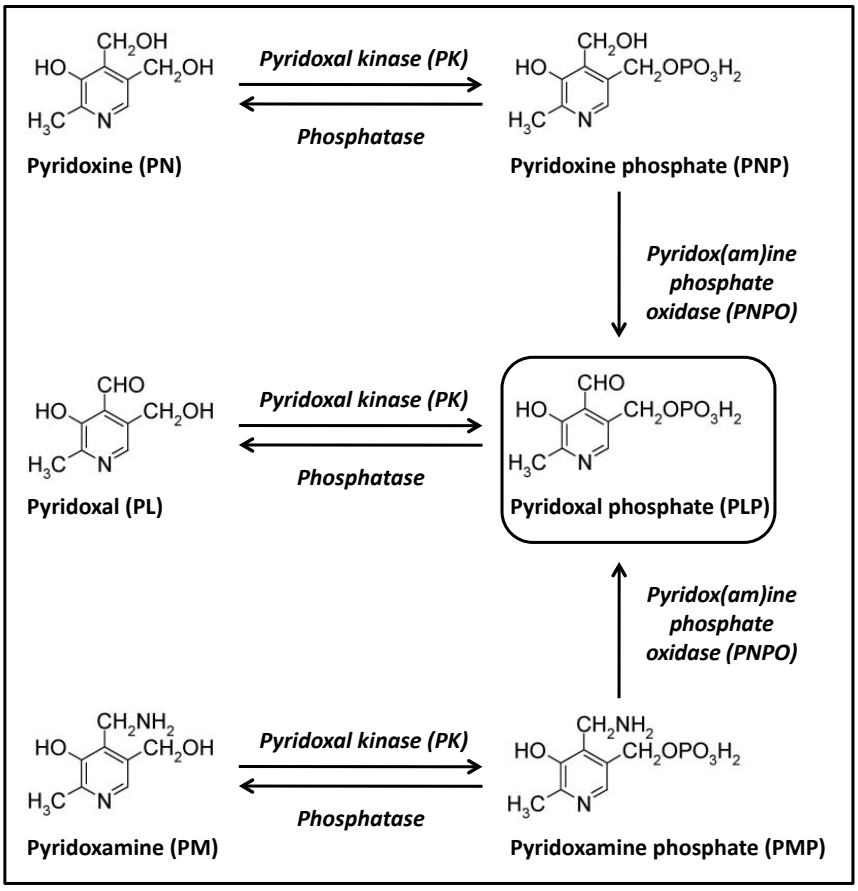

Figure 1 The different vitamin B6 vitamers and their intracellular conversions.

phosphate (PLP), the active form. [Clayton (2006)] (Figure 1) PA is the major degradation product of vitamin B6 which is excreted in urine. [Bender (2005)]

PLP is well-known for its functions as co-factor in a large number of essential enzymatic reactions in the central nervous system, where it mainly catalyses amino acid and neurotransmitter metabolism. PLP plays an important role in the biosynthesis of dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), D-serine and histamine. [Clayton (2006)] Although vitamin B6 deficiency is a rare condition, it gives rise to serious neurological symptoms, of which seizures are the most distinctive. In addition, low plasma PLP levels have been associated with depression [Hvas et al (2004)] and Alzheimer's disease [Miller et al (2002)] [Mulder et al (2005)] [Mulder et al (2007)]. In 2009, Elstner et al reported a polymorphism in the *pyridoxal kinase* gene which might be associated with the risk of Parkinson's disease [Elstner et al (2009)].

Vitamin B6 deficiency may arise from malnutrition, malabsorption of B6 vitamers from the diet, certain endogenous and exogenous nucleophiles (which inactivate PLP) or due to drugs which influence enzymes involved in PLP metabolism. [Clayton (2006)] [Footitt et al (2011)] Inherited vitamin B6 deficiency is found in patients with antiquitin deficiency, an autosomal recessive disorder of cerebral lysine degradation caused by mutations in the *ALDH7A1* gene [Mills et al (2006)], in PNPO deficiency, a disorder caused by mutations in the *PNPO* gene [Mills et al (2005)] and in hyperprolinaemia type II [Walker et al (2000)]. Antiquitin deficiency is the major cause of pyridoxine dependent epilepsy (PDE) and together with PNPO deficiency it warrants vitamin B6 supplementation to overcome the intractable seizures seen in these conditions. Antiquitin deficiency can be effectively treated by administration of pyridoxine whereas in PNPO deficiency, PL or PLP needs to be administered.

Antiquitin or PNPO deficiency cannot be reliably diagnosed in plasma since biochemical abnormalities are not always present. In cerebrospinal fluid (CSF) of patients affected with antiquitin [Mills et al (2010)] and PNPO deficiencies [Mills et al (2005)], elevated levels of threonine, 3-methoxytyrosine (L-dopamine metabolite) and glycine have been found. These observations are explained by a decreased activity of the responsible PLP-dependent enzymes due to a lack of PLP (threonine dehydratase, aromatic L-amino acid decarboxylase (AADC) and enzymes involving the glycine cleavage system, respectively). In addition, concentrations of the dopamine metabolite homovanillic acid (HVA) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) are decreased in CSF of PNPO-deficient patients [Mills et al (2005)], probably the result of a decreased AADC activity. The

abovementioned changes in CSF amino acid and neurotransmitter metabolite profiles are not observed in all PNPO-deficient patients. [Mills et al (2005)] [Hoffmann et al (2007)] [Khayat et al (2008)] In patients with antiquitin deficiency, CSF levels of pipecolic acid [Plecko et al (2005)] and α-aminoadipic semialdehyde (α-AASA) [Mills et al (2006)] are increased. However, antiquitin deficiency is likely not the only cause of PDE. [Kanno et al (2007)] [Schmitt et al (2010)] In addition, Veerapandiyan et al describe a case of PLP dependent epilepsy in which no *PNPO* gene mutations were found [Veerapandiyan et al (2011)]. Thus, disturbances of vitamin B6 metabolism may be missed by biochemical profiling of the secondary effects of vitamin B6 deficiency. Direct analysis of vitamin B6 vitamers in CSF may overcome these diagnostic limitations.

In plasma, B6 vitamer concentrations have been determined using different high performance liquid chromatography (HPLC) based methods. [Bates et al (1999)] [Marszałł et al (2009)] [Midttun et al (2007)] [Midttun et al (2009)] [Sassi et al (2002)] [Shephard et al (1987)] [Talwar et al (2003)] The number of methods for quantification of vitamin B6 in CSF is limited and methods are based on HPLC with fluorescence detection [Footitt et al (2011)] [Ormazabal et al (2008)] or radioactive tyrosine decarboxylase assays [Shin et al (1984)]. To our knowledge, B6 vitamer reference values in CSF have been reported for PLP only whereas CSF concentrations of the B6 vitamers PL, PM(P), PN(P) and PA have not yet been described. Information on all vitamin B6 vitamers is necessary to get more insight into its metabolism as has also been suggested by Mills et al [Mills et al (2005)]. Insight in vitamin B6 metabolism might not only enhance our insight into the pathophysiology of disorders associated with vitamin B6 deficiency, but might eventually lead to therapeutic strategies.

We present the development and validation of a rapid, sensitive and accurate method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF by ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS), using minimal CSF volumes and being suitable for implementation in a routine diagnostic setting. We established concentrations of CSF B6 vitamers in 20 subjects. In addition, CSF samples of two patients on pyridoxine supplementation were measured to demonstrate the applicability of our method.

MATERIALS AND METHODS

Reagents

PL-hydrochloride (≥ 99%), PM-dihydrochloride (≥ 98%), PN (≥ 98%), PA (≥ 99%), PLP-monohydrate (≥ 97%), PMP (≥ 98%) and heptafluorobutyric acid (HFBA, ≥ 99.5%) were

obtained from Sigma-Aldrich (Steinheim, Germany). PNP was kindly provided by C.J. Argoudelis, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign (Urbana, Illinois). PL-hydrochloride-D_3 (99%), PN-hydrochloride-$^{13}C_4$ (99%), PA-D_2 (98%) and methyl-D_3-PLP (97%) were purchased from Buchem bv (Apeldoorn, The Netherlands). Trichloroacetic acid (TCA, >99%) and acetic acid (99-100%) were obtained from Merck (Darmstadt, Germany). Acetonitrile (ULC-MS) was purchased from Biosolve (Valkenswaard, The Netherlands).

Standard solutions (B6 vitamers and internal standards (IS's)) and calibration curve

Stock solutions (μM) of vitamin B6 compounds were prepared for PL (6330), PM (523), PN (674), PA (1802), PLP (2048), PMP (2176) and PNP (1605). A ten point calibration curve was prepared by diluting these stock solutions in a mixture (end concentration (nM) for PL (317), PM (26), PN (34), PA (90), PLP (102), PMP (54) and PNP (40)).

A ten point high-range calibration curve was prepared by diluting stock solutions of vitamin B6 compounds in a mixture (end concentration (nM) for PL (6330), PM (523), PN (674), PA (1802), PLP (2048), PMP (1088) and PNP (803)).

Stock solutions (μM) of stable isotope labeled B6 vitamers were prepared for PL-D_3 (2372), $^{13}C_4$-PN (2242), PA-D_2 (2970) and PLP-D_3 (1320). An internal standard (IS) working solution was prepared by diluting these stock solutions in a mixture (end concentrations (nM) for PL-D_3 (47), $^{13}C_4$-PN (9), PA-D_2 (30) and PLP-D_3 (132)).

All stock solutions and subsequent dilutions were prepared in TCA (50 g L^{-1} Milli-Q water) and stored at -80°C.

Quality control samples (QC's)

CSF material of at least 25 random subjects was pooled and B6 vitamers were spiked to achieve three different concentration levels (QC 1, QC 2 and QC 3). For QC 1, spiked levels (nM) were 8 (PLP) and 5 (PMP). PL, PM, PN and PA were not spiked to QC 1. For QC 2, spiked levels (nM) were 20 (PL), 8 (PM), 5 (PN), 9 (PA), 28 (PLP) and 10 (PMP). For QC 3, spiked levels (nM) were 190 (PL), 18 (PM), 10 (PN), 35 (PA), 73 (PLP) and 40 (PMP). QC samples were divided into small portions and stored at -80°C until analysis.

Cerebrospinal fluid (CSF) samples

CSF from lumbar puncture was obtained of 20 subjects (age eight months to 16.3 years; 14 boys and six girls) who were investigated for developmental delay (*n*=14), movement

disorder (*n*=2) or both (*n*=4). Samples were provided by the Sylvia Tóth Center and archived at the Department of Metabolic Diseases (both Wilhelmina Children's Hospital, University Medical Center (UMC) Utrecht, The Netherlands). CSF was collected by means of a routine standardized procedure in which six separate fractions (0.5, 1, 1, 1.5, 1-2 and 2 mL, respectively) are immediately put on ice at the bedside. In this procedure, fraction IV is always protected from light and stored at -80°C right after collection. This fraction was used to establish B6 vitamer concentrations. Exclusion criteria were CSF erythrocytes >100 mL^{-1}, CSF leukocytes >10 mL^{-1} [Fuchs et al (2006)] and CSF protein >0.40 mg mL^{-1}, preventing sample contamination through injury of the blood-CSF barrier. CSF samples of patients with acute infections and/or antibiotic treatment, human immunodeficiency virus, auto-immune diseases, neoplastic disorders, schizophrenia, epilepsy and/or anti-epileptic drug treatment, specific other drug treatment regimes (anti-tuberculosis drugs, corticosteroids, Levo-dopa [Footitt et al (2011)]), vitamin B6 supplementation, established (neuro-)metabolic or other chronic disorders (such as malabsorption) and patients without sufficient information were all excluded.

From random patients who underwent lumbar puncture (*n*=5), B6 vitamers were analyzed in CSF fractions II, IV and VI to study a possible concentration gradient as described for monoamine metabolites [Kruesi et al (1988)]. In addition, CSF of two patients (A and B) on pyridoxine supplementation was investigated to study the effects of vitamin B6 therapy on CSF vitamers (A: age 19 days, pyridoxine dosage 30 mg kg^{-1} day^{-1}; B: age 6.25 years, pyridoxine dosage 27 mg kg^{-1} day^{-1}). Signed informed consent was obtained for all CSF samples, which were blinded before analysis. Approval by the Medical Ethics Committee of the UMC Utrecht was obtained.

Sample and calibration curve preparation

For analysis of B6 vitamers in CSF, 60 µL of IS working solution was added to 60 µL of CSF in an Eppendorf tube. After mixing for 5 sec, samples were protected from light and placed at room temperature for 15 min. After centrifugation (16.060 x g for 5 min), supernatants were transferred to a 96 wells plate and analyzed by UPLC-MS/MS. Samples were protected from light and were kept at 15°C during analyses.

Calibration curve standards were diluted (1:1 *v/v*) with IS working solution and analyzed with each series of CSF samples for B6 vitamer quantification.

Instruments

A Xevo-TQ MS triple quadropole mass spectrometer with an electrospray ionisation (ESI) source and an Acquity UPLC (Waters, Manchester, UK) were used. Masslynx software V4.1 (Waters, Manchester, UK) was used to control the instrument and for data acquisition.

Chromatographic and mass spectrometric conditions

Analytical UPLC columns with different chemistries (Acquity BEH C18, Acquity BEH C8, Acquity BEH Amide (all 1.7 µm, 2.1x100mm) and Acquity HSS-T3 (1.8 µm, 2.1x100mm) (Waters, Massachusetts, USA)) were tested. Selection parameters were retention stability, resolution and peak shape of the different B6 vitamers. Optimal results were obtained using an Acquity HSS-T3 guard column (1.8 µm, 2.1x5mm) with an Acquity HSS-T3 analytical column (Waters, Massachusetts, USA). Column temperature was kept at 25°C and the injection volume was 10 µL. A two step (linear) gradient of 3.5 minutes at a flow rate of 0.4 mL min^{-1} between solvent A (650 mM acetic acid with 0.01% HFBA) and solvent B (100% acetonitrile) was used. The gradient started with 100% solvent A. Between 0.5 min and 2.0 min it changed to 80% solvent A. In 0.1 min the gradient switched to 100% solvent B which was maintained during the next 0.2 min. A direct switch to 100% solvent A was made and 1.2 min was used for column equilibration.

The MS was operated in the positive ESI mode. A capillary voltage of 0.5 kV and a cone voltage of 18 V were used. Source and desolvation temperatures were 150°C and 550°C, respectively. Ultra-high purity nitrogen was used for cone gas (18 L h^{-1}), desolvation gas (1150 L h^{-1}) and nebulising gas (100 L h^{-1}). For collision induced dissociation (CID), ultra-pure Argon (0.003 mbar) at a flow rate of 0.20 mL min^{-1} was used. Multiple reaction monitoring (MRM) transition of each vitamin B6 compound was optimized by direct infusion. Unit resolution in the first and third quadropole was used. Table 1 shows mass transitions, cone voltages and collision energies of individual vitamin B6 compounds and stable isotopes that were used as IS's. Since no analogue stable isotopes are available for PM, PMP and PNP, PL-D_3 and PLP-D_3 were used to calculate the area ratio for these B6 vitamers. See Figure 2 for a MRM chromatogram (QC 2) of the different vitamin B6 compounds and isotope labeled internal standards.

Table 1 Mass transitions, cone voltages, collision energies and internal standards of the different B6 vitamers and stable isotopes.

Compound	Parent ion $[M+H]^+$	Daughter ion	Cone (V)	CE (eV)	IS
PL	168.1	150.1	15	12	PL-D_3
PM	169.1	134.1	19	22	PL-D_3
PN	170.1	134.1	18	20	PN-$^{13}C_4$
PA	184.1	148.1	18	18	PA-D_2
PLP	248.1	150.1	25	14	PLP-D_3
PMP	249.1	232.1	24	14	PLP-D_3
PNP	250.1	134.1	25	20	PLP-D_3
PL-D_3	171.1	153.1	15	12	
PN-$^{13}C_4$	174.1	138.1	18	20	
PA-D_2	186.1	150.1	18	18	
PLP-D_3	251.1	153.1	25	14	

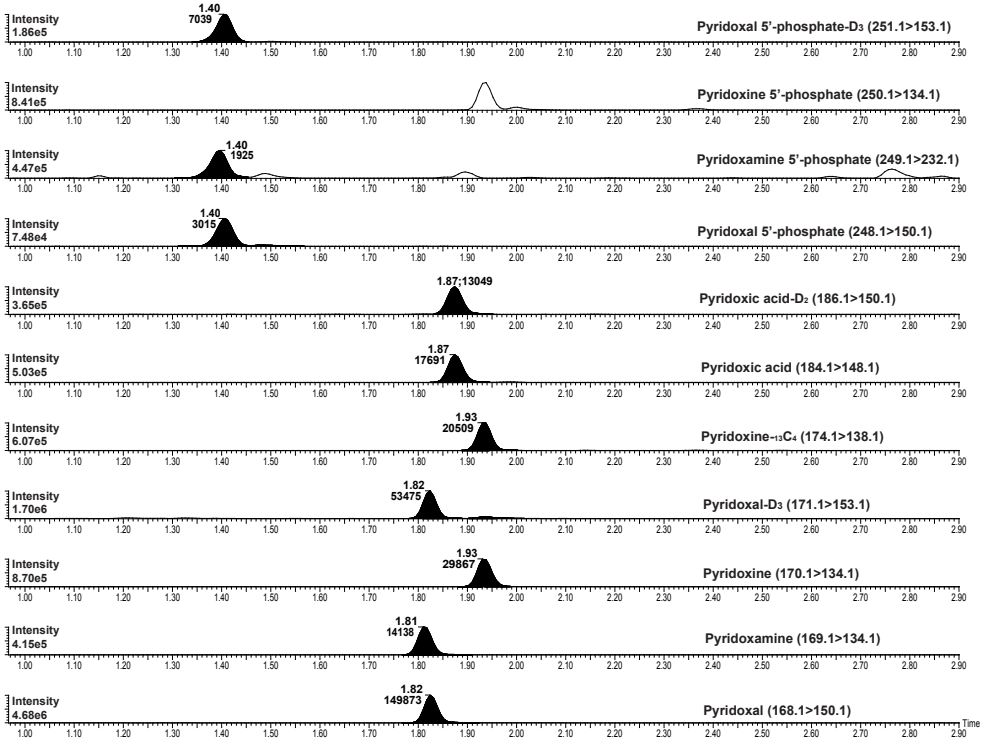

Figure 2 Chromatogram of MRM transitions of vitamin B6 compounds (PL, PM, PN, PA, PLP, PMP and PNP) and isotope labeled internal standards (PL-D_3, $^{13}C_4$-PN, PA-D_2 and PLP-D_3) in CSF (QC 2).

Method validation

QC 1, QC 2 and QC 3 were used for method validation. Precision (intra- and inter-assay variation), accuracy, sensitivity (limit of detection (LOD) and limit of quantification (LOQ)), linearity, recovery, matrix effect, short-term stability, freeze-thaw stability and exposure to light were tested for all vitamin B6 compounds during assay validation.

Intra- and inter-assay (*n*=10 and *n*=20, respectively) variations were studied by analyzing QC 1, QC 2 and QC 3.

Standard addition experiments were performed to test assay accuracy. A random CSF pool was spiked with five different B6 vitamer levels (range of calibration curve) and the slope was compared with the slope obtained from the calibration curve.

QC 3 (*n*=3) was used to determine LOD and LOQ for all vitamin B6 compounds (signal-to-noise ratios (S/N) 3 and 10, respectively).

Linearity was evaluated using the high-range calibration curve. Standards of this curve were diluted (1:1 *v/v*) with IS working solution.

Recovery experiments were performed (*n*=2) in QC 1, for both unlabeled and isotope labeled vitamin B6 compounds, by spiking before and after protein precipitation. For the unlabeled vitamin B6 compounds, 5 concentration levels (end concentration (nM) for PL (327), PM (129), PN (192), PA (137), PLP (153) and PMP (54)) were added. For the isotope labeled compounds, IS working solution was added. Recovery was tested by comparing peak areas of individual unlabeled and isotope labeled vitamin B6 compounds before and after protein precipitation.

Matrix effect was determined by comparing peak areas of individual isotope labeled compounds in standards of the calibration curve with peak areas of individual isotope labeled compounds in CSF samples (QC 1, QC 2 and QC 3 (*n*=3)).

Short-term stability was tested by measuring vitamin B6 compounds in a prepared calibration curve together with 10 prepared random CSF samples at four time points during 11 days. During analyses and sample storage, samples were protected from light. Between analyses, calibration curve and samples were kept at 4°C.

Two random CSF samples (*n*=3) were used to test stability of the vitamin B6 compounds during 1, 3, 5 and 10 freeze-thaw cycles. Samples were thawed at room temperature and kept at room temperature for one hour, protected from light.

QC 1, QC 2 and QC 3 (*n*=2) and calibration curve standards were tested for the influence of daylight during 0, 4 and 10 hours at room temperature before sample preparation. Slopes obtained from the calibration curves were compared. For QC 1, QC 2 and QC 3 the analyzed B6 vitamer concentrations were compared. Additional experiments to study the influence

of light at different temperatures (exposure to light compared with darkness during 0, 4 and 10 hours at room temperature versus ice) were performed.
Cross-talk was studied by spiking individual B6 vitamer compounds (*n*=2) to randomly pooled CSF. Spiked concentrations (nM) were 295 (PL), 141 (PM), 195 (PN), 140 (PA), 205 (PLP) and 97 (PMP). Concentrations of all B6 vitamers were compared in spiked and unspiked pooled CSF.

Statistical analysis

Analyse-it v2.23 (Analyse-it Software Ltd., Leeds, UK) was used to check for normal distribution (Anderson-Darling A^2) and to determine calibration curve linearity for the vitamin B6 compounds (non-linear specification of 5%).

RESULTS

Chromatographic and mass spectrometric conditions

During method development a contribution of PN (*m/z* 170.1 > 134.1) was observed in the MRM trace of PL-D_3 (*m/z* 171.1 > 153.1). Maximum resolution between PN and PL-D_3 was achieved using the Acquity HSS-T3 column. Total run time was 3.5 min. Retention times were 1.82 min for PL, 1.81 min for PM, 1.93 min for PN, 1.87 min for PA and 1.40 min for PLP, PMP and PNP (Figure 2).

Method validation

Because spiked amounts of PNP were not detectable in QC 2 and QC 3, intra- and inter-assay CV's, LOD and LOQ could not be established. Accuracy deviation and ion-suppression were too high for PNP (data not shown). Given these poor results, PNP cannot be properly quantified with the described method.

Table 2 shows analyzed concentrations (nM) and intra- and inter-assay coefficients of variation (CV's) for all vitamin B6 compounds in QC 1, 2 and 3. Intra-assay CV's varied between 2.3% and 19.1%. Inter-assay CV's varied between 4.5% and 19.3%. To investigate precision of quantification of decreased PLP concentrations, we determined intra- and inter-assay CV's in a CSF sample with a PLP concentration of 1.9 nM. These were 17.7% and 16.9%, respectively.

Table 2 Precision of the described UPLC-MS/MS method (inter- and intra-assay variation) with mean B6 vitamer concentrations in QC 1, QC 2 and QC 3.

	Concentration (nM)			Intra-assay CV (%), *n*=10			Inter-assay CV (%), *n*=20		
Compound	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
PL	11.0	31.9	185.1	3.9	2.7	2.3	8.9	5.1	4.5
PM	1.8	11.9	23.6	17.9	6.0	4.3	19.3	15.1	13.5
PN	0.8	3.8	8.5	19.1	9.2	6.0	18.4	5.9	4.6
PA	1.8	12.7	47.6	10.5	6.0	3.0	16.7	4.5	4.8
PLP	8.7	26.2	64.7	8.2	6.2	4.5	7.1	6.3	5.2
PMP	7.4	13.4	51.6	14.0	9.3	8.1	6.9	10.3	6.7

Accuracy with a deviation of <10% was found for PL, PN, PA and PLP (Table 3). The B6 vitamers PM and PMP showed higher deviations (20% and 39%, respectively).

For each B6 vitamer, LOD and LOQ are shown in Table 3. LOD varied between 0.01 nM (PM and PN) and 1.61 nM (PMP). LOQ varied between 0.03 nM for PN and 5.37 nM for PMP.

Upper limits of linearity for all B6 vitamers are shown in table 3.

Recoveries were 103% for PL, 108% for PM, 96% for PN, 103% for PA, 104% for PLP and 99% for PMP. For the labeled vitamin B6 compounds, recoveries were comparable: 94% for PL-D_3 and 93% for $^{13}C_4$-PN and PA-D_2. PLP-D_3 showed a recovery of 96%.

While studying matrix effects, a signal reduction of 25% was found for PL-D_3 whereas signal reductions were 44%, 24% and 87% for $^{13}C_4$-PN, PA-D_2 and PLP-D_3, respectively.

Short-term stability was tested for PL, PA and PLP in prepared CSF samples. The other B6 vitamers showed a concentration <LOQ in the analyzed CSF samples and could therefore not be included. After 11 days, no significant decrease was observed.

Stability tests during 10 freeze-thaw cycles showed no significant changes for PL, PN and PA compared to their respective inter-assay variations. Although PM showed no significant change after 3 cycles, a decrease of 18% and 34% was found after 5 and 10 cycles, respectively. PLP however already showed a decrease of 49% after 3 freeze-thaw cycles and a decrease of 86% was found after 10 cycles. For PMP stability could not be tested because of a concentration <LOQ in the used CSF samples.

Table 3 Accuracy, sensitivity (LOD and LOQ) and linearity (upper limits) of B6 vitamers analyzed with the described UPLC-MS/MS method. B6 vitamer concentrations in CSF of 20 subjects (age eight months to 16.3 years) and in CSF of two patients (A and B) on pyridoxine supplementation.

		Concentration (nM)						
Compound	Accuracy (%)	LOD	LOQ	Linearity	Mean	Range	*Patient A*	*Patient B*
PL	105	0.46	1.54	1266	27.5	14.8 - 42.5	*3776*	*1194*
PM	80	0.01	0.04	105	0.30	0.1 - 0.5	*29.6*	*6.0*
PN	97	0.01	0.03	270	NA	<0.03[a]	*18881*	*353*
PA	108	0.03	0.09	90	1.5	0.09[a] - 4.1	*104*	*81.7*
PLP	95	0.12	0.38	205	20.2	8.8 - 42.0	*41.2*	*39.9*
PMP	139	1.61	5.37	435	NA	<5.4[a]	*<5.4[a]*	*<5.4[a]*

[a] Determined LOQ of this vitamin B6 compound. NA: not applicable.

Concentrations of all vitamin B6 compounds (PL, PM, PN, PA, PLP, PMP) in the calibration curve showed no significant change after 4 hours of exposure to light at room temperature. After 10 hours of exposure to light, only PA and PLP showed a significant decrease in concentration (31% and 50%, respectively).

CSF samples on the contrary (mean of QC 1, QC 2 and QC 3) already showed a significant decrease after 4 and 10 hours of exposure to light at room temperature for PL, PM, PN, PLP and PMP (Figure 3). PA showed no significant change. After 10 hours at room temperature a decrease range of 42% for PN to 70% for PLP was found (Figure 3). Additional experiments testing temperature-dependent stability (exposure to light at room temperature versus ice) showed light dependence and no influence of temperature. In line with this, exposure to darkness during 4 and 10 hours at room temperature did not result in a significant change in any of the vitamin B6 compounds (data not shown).

Furthermore, no cross-talk was observed since spiking of individual B6 vitamers did not significantly change other B6 vitamer concentrations.

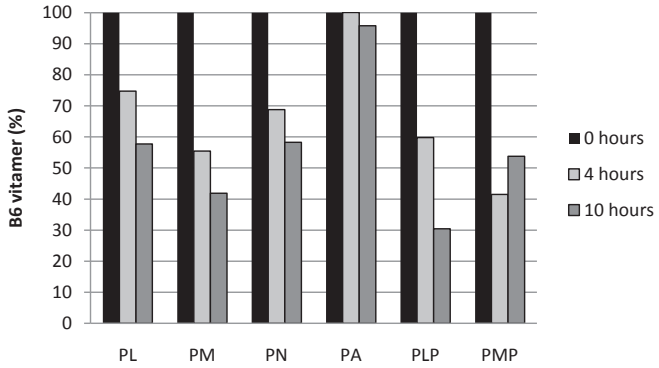

Figure 3 Influence of light exposure during 0, 4 and 10 hours at room temperature for the B6 vitamers PL, PM, PN, PA, PLP and PMP (mean of QC 1, QC 2 and QC 3; *n*=2).

Cerebrospinal fluid (CSF) samples

Concentration ranges in human CSF (nM) were established for PL (14.8 – 42.5), PM (0.1 – 0.5), PA (0.09 – 4.1) and PLP (8.8 – 42.0) (Table 3). For the B6 vitamers PN and PMP, CSF concentrations were <LOQ. Vitamin B6 concentrations showed a normal distribution and no age and gender correlations were observed in this dataset.

No significant differences compared to inter-assay variations in B6 vitamer concentration (PL, PM, PN, PA and PLP) were present between the different CSF fractions (II, IV and VI) of random lumbar punctures, indicating that there is no rostrocaudal gradient of B6 vitamers in human CSF. PMP concentration was <LOQ.

In CSF of two patients on pyridoxine supplementation (Table 3), highly increased concentrations of the B6 vitamers PL, PM, PN and PA were observed. PLP concentrations were at the upper limit of the established concentration range. In both patients, PMP concentrations remained <LOQ.

DISCUSSION

In the present study we show for the first time the development of a rapid, sensitive and accurate UPLC-MS/MS method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF using minimal CSF volumes (60 µL). The described method is based on the HPLC method in plasma by Midttun et al with several modifications [Midttun et al (2009)].

Information on vitamin B6 concentrations in CSF is limited in both healthy individuals and in patients with epilepsy, other neurological disease or disorders of vitamin B6 metabolism.

Low levels of PL and PLP have been reported in CSF of only a few patients affected with PNPO deficiency (for PL *n*=3 [Mills et al (2005)]; for PLP *n*=4 [Mills et al (2005)] [Ruiz et al (2008)]) and antiquitin deficiency (for PLP, *n*=2 [Footitt et al (2011)]). However, no data on PN(P), PM(P) and PA concentrations in CSF have been published. Since our method shows intra- and inter-assay CV's of 17.7% and 16.9% for PLP at a concentration of 1.9 nM, levels below the concentration range of 8.8 – 42.0 nM can be quantified in human CSF. The same accounts for PL. Unfortunately, we did not have CSF samples of PNPO or antiquitin deficient patients at our disposal.

Concentrations of the active co-factor PLP in CSF were comparable with those reported previously by Footitt et al and Ormazabal et al, who additionally showed an inverse correlation of CSF PLP with age [Footitt et al (2011)] [Ormazabal et al (2008)]. Our sample size was too small to allow for meaningful correlation studies.

No concentrations for PL, PM and PA have been reported in CSF before. Since we used a highly specific UPLC-MS/MS method with stable isotope labeled internal standards, our CSF B6 vitamer measurements are very accurate.

The present study also shows that vitamin B6 concentrations are not affected by a rostrocaudal gradient, meaning that vitamers can be measured in a random CSF sample. However, it must be warranted that vitamin B6 is determined in CSF fractions which were protected from light at the bedside and frozen immediately after collection to prevent degradation by light and multiple freeze-thaw cycles, respectively.

In plasma, vitamin B6 vitamer concentrations have been described by Midttun et al. In 94 healthy subjects aged 11-93 (median 56) years, plasma PL(P), PM, PN and PA were determined using an HPLC-MS/MS method. Whereas we found CSF B6 vitamer concentrations of PL>PLP>PA>PM, Midttun et al described plasma B6 vitamer concentrations of PLP>PA>PL [Midttun et al (2009)]. This is in agreement with the studies of Marszałł et al and Talwar et al who also showed plasma PLP>PA>PL [Marszałł et al (2009)] [Talwar et al (2003)]. Thus, clear differences in vitamin B6 profiles exist between plasma and CSF. In CSF, PL is the B6 vitamer with the highest concentration whereas in plasma PLP and PA are higher compared to PL. In addition, in CSF PM is present in quantifiable amounts whereas both Midttun et al and Marszałł et al could not detect PM in plasma [Midttun et al (2009)] [Marszałł et al (2009)].

Interestingly, the described method allowed us to analyze vitamin B6 vitamers in CSF of two patients on pyridoxine supplementation. Highly elevated concentrations of PN (18881 and 353 nM, respectively) and PL (up to approximately 90 times higher) were found. Surprisingly, PLP concentrations were at the upper limit of the established concentration range (41.2 and 39.9 nM, respectively). In addition, PM and PA were at least approximately

10 and 20 times higher. These findings clearly demonstrate that PN passes the blood-brain and/or blood-CSF barrier.

CONCLUSION

We present an innovative method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF samples. Our method is suitable for implementation in a routine diagnostic setting since decreased PL and PLP concentrations can be detected. In addition, effects of vitamin B6 therapy on CSF vitamers can be monitored. Analyzing vitamin B6 vitamers will increase our knowledge of vitamin B6 metabolism and CSF vitamer concentrations in health and disease.

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CHAPTER 3

Vitamin B6 vitamer concentrations in cerebrospinal fluid differ between preterm and term newborn infants

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ABSTRACT

Vitamin B6 plays a pivotal role in brain development and functioning. Differences in vitamin B6 homeostasis between preterm and term newborn infants have been reported. Our objective was to investigate whether B6 vitamers in cerebrospinal fluid (CSF) of preterm and term newborn infants are different.

B6 vitamer concentrations were determined in 69 CSF samples of 36 newborn infants (26 born preterm and 10 born term) by ultra performance liquid chromatography-tandem mass spectrometry. CSF samples, taken from a subcutaneous intraventricular reservoir, were bedside frozen and protected from light.

Concentrations of pyridoxal (PL), pyridoxal phosphate (PLP), pyridoxic acid (PA) and pyridoxamine (PM) in preterm newborns (postmenstrual age 30-37 weeks) were at least twice as high as in older newborns (postmenstrual age ≥42 weeks). Pyridoxine and pyridoxamine phosphate concentrations were below limits of quantification in all newborns. In CSF of two very preterm newborns (postmenstrual age <30 weeks), significant amounts of pyridoxine were present besides high concentrations of PL, PA and PM, whereas PLP concentrations were relatively low. B6 vitamers in CSF were positively correlated, especially PA, PLP and PL.

In conclusion, PL, PLP, PA and PM are present in CSF of newborn infants and concentrations are strongly dependent on postmenstrual age. Our results indicate that vitamin B6 homeostasis in brain differs between preterm and term newborns. These results should be taken into account for diagnosis and treatment of epilepsy and vitamin B6 deficiency in newborn infants.

INTRODUCTION

Vitamin B6 is a water-soluble and for humans essential nutrient. It comprises different vitamers: the alcohol pyridoxine (PN), the aldehyde pyridoxal (PL), the amine pyridoxamine (PM) and their phosphate esterified forms. Pyridoxic acid (PA) is the major degradation product of vitamin B6 which is excreted in urine [Bender (2005)]. The active form of vitamin B6, pyridoxal phosphate (PLP), is well-known as a co-factor to a large number of essential enzymatic reactions in the central nervous system which catalyze amino acid and neurotransmitter metabolism.

Prior to transport across membranes of brain cells and choroid plexus, phosphorylated B6 vitamers must be hydrolysed by a membrane-bound alkaline phosphatase [Spector and Greenwald (1978)] [Waymire et al (1995)]. Intracellular (re-)phosphorylation by pyridoxal kinase is followed by a pyridox(am)ine phosphate oxidase - mediated conversion of pyridoxine phosphate (PNP) and pyridoxamine phosphate (PMP) into PLP [Clayton (2006)]. (Figure 1) Brain cells only release the unphosphorylated B6 vitamers after hydrolysis by a specific phosphatase, whereas choroid plexus releases phosphorylated as well as unphosphorylated B6 vitamers into cerebrospinal fluid (CSF) [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies].

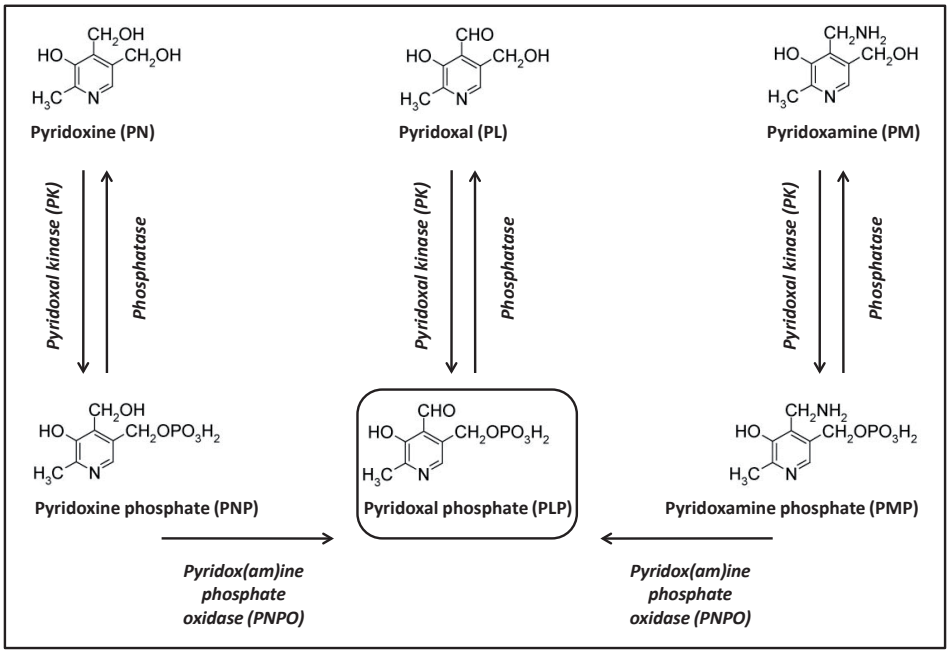

Figure 1 The different vitamin B6 vitamers and their intracellular conversions.

PLP is important for the biosynthesis of dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), histamine [Clayton (2006)] and D-serine [Fuchs et al (2005)], which suggests a crucial role in brain development and functioning. Several studies performed in rats indeed have shown lower brain weights [Morré and Kirksey (1978)], impaired neuromotor development [Alton-Mackey and Walker (1973)] and seizures [Alton-Mackey and Walker (1973)] [Groziak and Kirksey (1987)] after prenatal induction and postnatal maintenance of dietary vitamin B6 deficiency. In addition, adverse effects on neurogenesis, neuronal longevity [Groziak and Kirksey (1987)], neuronal differentiation [Groziak and Kirksey (1990)] and synaptogenesis [Groziak and Kirksey (1990)] [Wasynczuk et al (1983)] have been described as well as reduced myelination [Morré and Kirksey (1978)] [Morré et al (1978)]. In humans, genetic vitamin B6 deficiency results in seizures, which can be accompanied by variable degrees of structural brain abnormalities and psychomotor retardation. Several autosomal recessive disorders are known to be causative [Stockler et al (2011)], such as antiquitin deficiency, a disorder of cerebral lysine degradation caused by mutations in the *ALDH7A1* gene [Mills et al (2006)] and pyridox(am)ine phosphate oxidase deficiency, caused by mutations in the *PNPO* gene [Mills et al (2005)]. Whereas seizures due to antiquitin deficiency are responsive to PN, PL(P) must be administered in case of PNPO deficiency. Other genetic causes of vitamin B6 deficiency are hyperprolinaemia type II [Walker et al (2000)] and hypophosphatasia (tissue non-specific alkaline phosphatase deficiency) [Waymire et al (1995)] [Whyte et al (1988)].

Since vitamin B6 is crucial for brain development and functioning, knowledge on vitamin B6 homeostasis in healthy newborn infants is clinically highly important. In this context, the studies of Raiten et al are interesting, as they reported low concentrations of PLP in serum of preterm newborn infants <30 weeks of gestational age (GA) (range 25-29 weeks; *n*=15) [Raiten et al (1991)]. Furthermore, they demonstrated absence of a serum PLP response in newborns <30 weeks of GA receiving intravenous pyridoxine up to a postnatal age of 28 days. This was in clear contrast to newborns ≥30 weeks of GA (range 30-40 weeks; *n*=13) in whom serum PLP concentrations were initially low, but increased significantly following pyridoxine supplementation. In preterm newborns ≤28 weeks of GA (*n*=9), supplementation of pyridoxal did not induce a serum PLP response either. [Raiten et al (1991)] In these studies, no information was obtained on brain homeostasis of vitamin B6.

The findings of Raiten et al triggered us to study vitamin B6 homeostasis in newborn infants. We used CSF samples, as they provide the only accessible reflection of brain homeostasis in humans. We compared B6 vitamers in CSF of preterm and term newborn infants.

SUBJECTS AND METHODS

We obtained 69 remnant CSF samples of 36 newborn infants (18 female, 18 male). Twenty-six infants were born preterm (<37 weeks of gestation) and 10 were born term (≥37 weeks of gestation). The youngest preterm infant was born at 26^{+0} weeks of gestation, whereas the oldest term infant was born at 41^{+1} weeks. Postnatal age at CSF withdrawal ranged from two to 153 days and postmenstrual age (postnatal age corrected for duration of pregnancy) ranged from 28^{+2} weeks to 53^{+5} weeks.

Newborn infants were admitted to the neonatal intensive care unit of the Wilhelmina Children's Hospital, University Medical Center (UMC) Utrecht, The Netherlands. Indication for CSF withdrawal was a post-hemorrhagic ventricular dilatation (*n*=33) or congenital hydrocephaly (*n*=3) with the necessity to remove CSF at regular intervals from a subcutaneous intraventricular reservoir to avoid or alleviate raised intracranial pressure. CSF samples were collected between 2003 and 2010. Immediately after withdrawal they were centrifuged (1200xg), protected from light and stored at -80°C. Parental informed consent was obtained to use remnants of clinically obtained CSF samples. Approval by the Medical Ethics Committee of the UMC Utrecht was obtained. B6 vitamer (PL(P), PM(P), PN and PA) concentrations were determined using a sensitive and accurate ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method with stable isotope labeled internal standards [Van der Ham et al (2012)].

Twenty-nine newborn infants were breast fed (with or without addition of breast milk fortifier (50 µg pyridoxine/100 mL)) and/or received (preterm) infant formula containing 40-120 µg pyridoxine/100 mL [Vermeer (2011)]. Seven newborns received parenteral nutrition (490 µg pyridoxine/kg body weight). None of the newborns was supplemented with additional vitamin B6. No information was available concerning maternal nutritional status and maternal vitamin B6 supplementation.

SPSS 15.0 (IBM Corporation, Somers, New York, USA) was used for statistical analysis. Since B6 vitamer concentrations and residuals did not show a normal distribution, non-parametric tests (Kruskal-Wallis and Mann-Whitney U with Bonferroni correction) were applied to study differences in median B6 vitamer concentrations and ratios between subgroups. Spearman's rho was used to describe correlations between individual B6 vitamers.

RESULTS

In CSF of newborn infants, PL, PLP, PA and PM were present, whereas PN and PMP were not detectable (limits of quantification 0.03 and 5.4 nM, respectively [Van der Ham et al (2012)]). Median B6 vitamer concentrations in CSF with their respective ranges are depicted in Table 1. Subgroups of postmenstrual age were defined by correcting postnatal age for duration of pregnancy (*n*=67): 30-37 weeks (*n*=25), 37-42 weeks (*n*=23; 74% born preterm) and ≥42 weeks (*n*=19; 90% born term). B6 vitamer concentrations in CSF of two very preterm newborn infants (A and B) with a postmenstrual age <30 weeks are reported separately (*n*=2).

Table 1 Vitamin B6 vitamer concentrations (ranges and medians) in CSF of newborn infants, divided into subgroups of postmenstrual age (*n*=67). In addition, ratios between individual B6 vitamers are shown and two very preterm newborns (A and B) are presented (*n*=2).

	Range (median) of B6 vitamer concentration (nM)				**Ratio between individual B6 vitamers (range (median))**	
Subgroup[1]	**PL[2]**	**PLP[2]**	**PA[2]**	**PM[3]**	**PA:PL[2]**	**PLP:PL[4]**
30 – 37 weeks (*n*=25)	46 – 226 (105)	28 – 170 (101)	6.0 – 73 (15)	0.5 – 3.6 (1.4)	0.08 – 0.49 (0.17)	0.35 – 2.51 (0.91)
37 – 42 weeks (*n*=23)	16 – 199 (102)	19 – 221 (106)	1.9 – 52 (13)	0.3 – 3.3 (1.0)	0.04 – 0.28 (0.15)	0.45 – 3.20 (1.02)
≥42 weeks (*n*=19)	14 – 103 (49)	8.0 – 76 (32)	0.9 – 11 (4.7)	0.3 – 1.4 (0.7)	0.02 – 0.33 (0.08)	0.15 – 1.92 (0.68)
Newborn A (28^{+2} weeks)	333	38	227	7.4	0.68	0.11
Newborn B (29^{+0} weeks)	238	57	275	4.6	1.16	0.24

[1] Postmenstrual age (37-42 weeks: 74% born preterm; ≥42 weeks: 90% born term); [2] p-value of difference between subgroups (Kruskal-Wallis test) <0.0005; [3] p-value of difference between subgroups (Kruskal-Wallis test) <0.0015; [4] p-value of difference between subgroups (Kruskal-Wallis test) >0.05 (not significant)

B6 vitamer concentrations in CSF were dependent on postmenstrual age (Table 1, Figure 2): concentrations in preterm newborns (postmenstrual age 30-37 weeks) were at least twice as high as in older newborns (postmenstrual age ≥42 weeks; 90% born term) ($p<0.0005$; Mann-Whitney U tests with Bonferroni correction (cut-off significance level $p=0.017$)). This difference was also observed between term newborns (postmenstrual age 37-42 weeks; 74% born preterm) and older newborns (age ≥42 weeks; $p<0.0005$ for PL, PLP and PA; $p=0.014$ for PM).

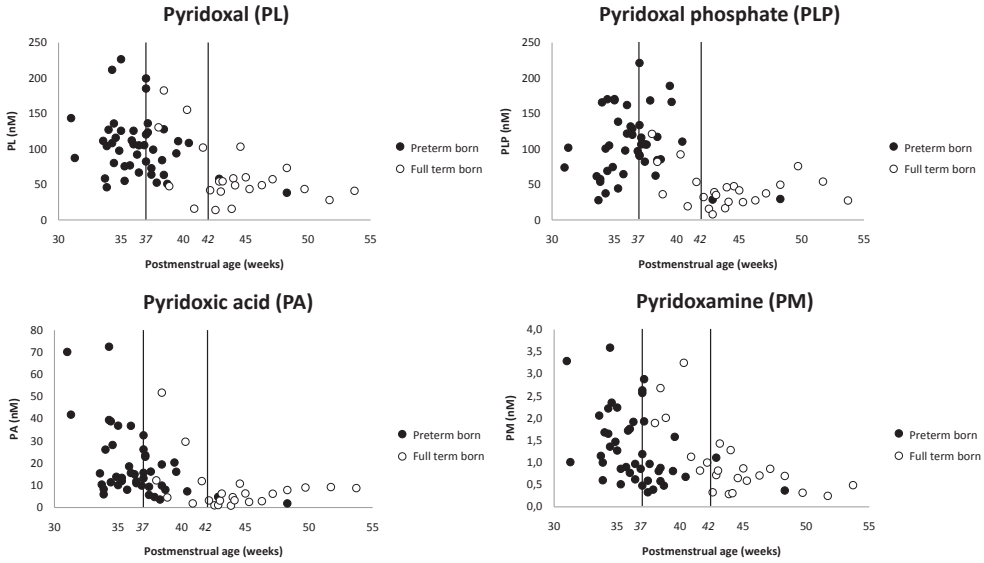

Figure 2 B6 vitamer concentrations (nM) in CSF of newborn infants divided into preterm or term birth with subgroups of postmenstrual age depicted by vertical lines (*n*=67).

In CSF of the two very preterm newborns (postmenstrual age for A 28^{+2} weeks and for B 29^{+0} weeks), significant amounts of PN (1.7 and 2.8 nM, respectively) were present besides high concentrations of PL, PA and PM. PLP concentrations however, were relatively low. (Table 1, Figure 3) Both these newborns received parenteral nutrition. Analysis of an additional CSF sample of newborn B, withdrawn at a postmenstrual age of 34 weeks, showed B6 vitamer concentrations consistent with values found in the subgroup of preterm newborns (postmenstrual age 30-37 weeks).

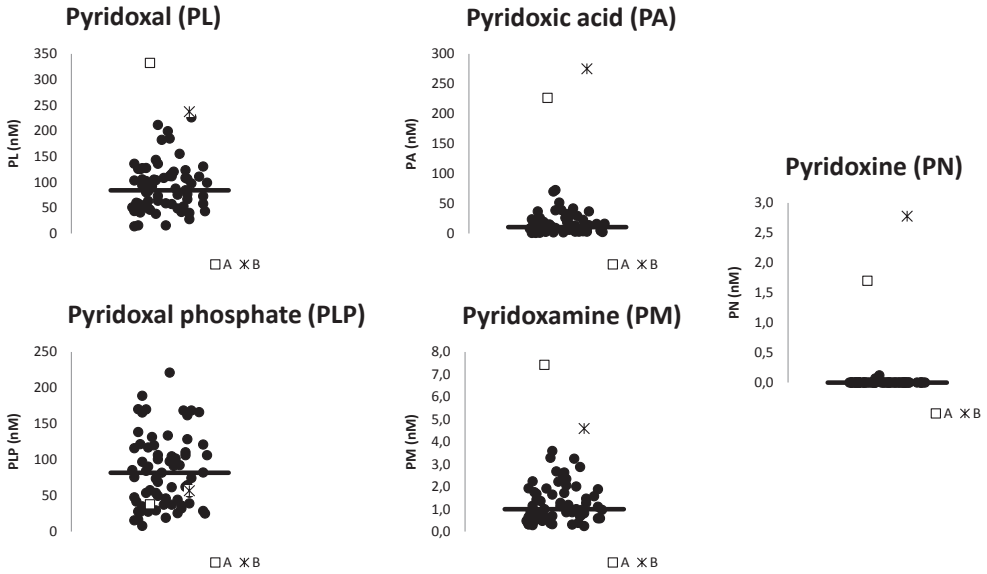

Figure 3 B6 vitamer concentrations (nM) in CSF of newborn infants A and B (postmenstrual age 28^{+2} and 29^{+0} weeks, respectively (*n*=2)), depicted in comparison with median B6 vitamer concentrations in newborn infants with a postmenstrual age >30 weeks (*n*=67).

B6 vitamer concentrations in CSF were positively correlated (*n*=67). (Table 2, Figure 4) A strong correlation was observed between the concentration of the active co-factor PLP, its direct precursor PL and the concentration of the degradation product PA (Spearman's rho 0.631 for PLP and PL; 0.849 for PA and PL, respectively; p<0.0005). The ratio between PA and PL was higher in preterm (postmenstrual age 30-37 weeks) and term (age 37-42 weeks) newborns compared to older newborns (age ≥42 weeks) (p<0.0005 and p=0.009, respectively; Mann-Whitney U tests with Bonferroni correction (cut-off significance level p=0.017)). The ratio between PLP and PL was not dependent on postmenstrual age. (Table 1) In both very preterm newborns A and B, correlations of PA, PLP and PL and ratios between these B6 vitamers were different compared to newborn infants with a postmenstrual age >30 weeks. (Table 1, Figure 4)

Table 2 Correlations (Spearman's rho) between individual B6 vitamers in CSF of newborn infants (*n*=67).

	PM	PA	PLP
PL	0.687 p<0.0005	0.849 p<0.0005	0.631 p<0.0005
PM		0.627 p<0.0005	0.297 p=0.015
PA			0.627 p<0.0005

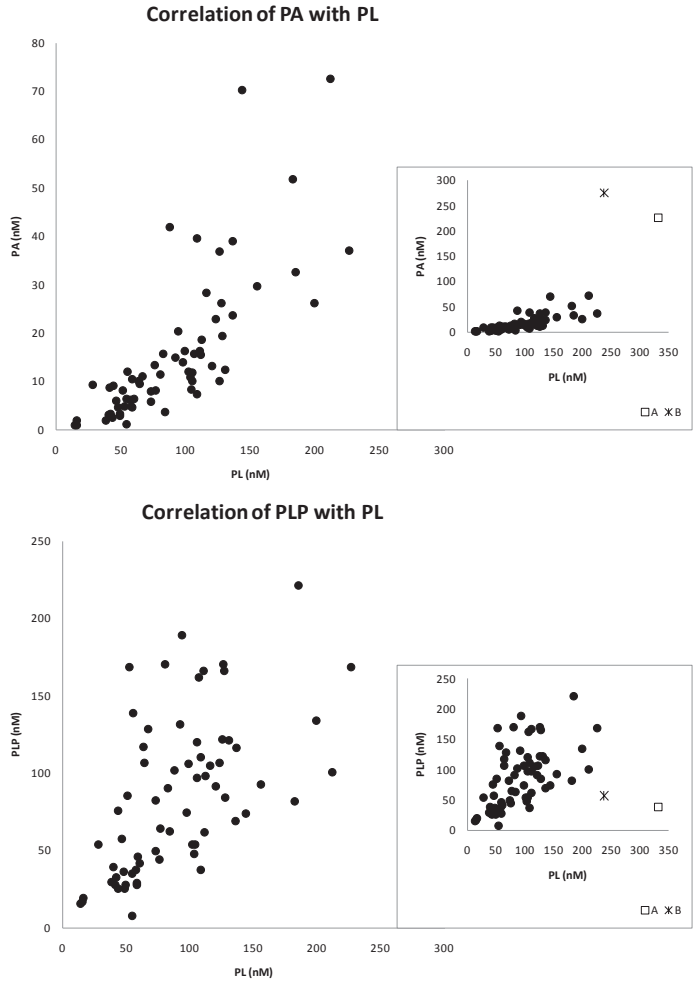

Figure 4 Correlations of the B6 vitamers PA (Spearman's rho 0.849) and PLP (0.631) with PL in CSF (p<0.0005; *n*=67), with an illustration of newborn infants A and B (*n*=2).

B6 vitamer concentrations were determined in 69 CSF samples of 36 newborn infants. Since the number of CSF samples of each newborn ranged from one to five at different postmenstrual ages, it is likely that subsequent CSF samples of an individual newborn belonged to the same subgroup. Exclusion of all subsequent CSF samples after initial CSF withdrawal from each newborn for each subgroup however, did not significantly change B6 vitamer concentration differences between subgroups (*n*=45). In fact, those individual newborns from whom multiple CSF samples were collected, all showed a decrease of B6 vitamer concentrations with postmenstrual age (data not shown).

Remarkably, in CSF of two newborns on parenteral nutrition (*n*=2), PN was present in quantifiable amounts (0.07 and 0.12 nM), whereas it was below limits of quantification (0.03 nM [Van der Ham et al (2012)]) in all other newborns with a postmenstrual age >30 weeks (*n*=65).

DISCUSSION

In CSF of newborn infants, we found B6 vitamer concentrations of PL>PLP>PA>PM, showing that vitamin B6 in CSF is mainly present as its active co-factor and direct precursor (PLP and PL, respectively). This is in agreement with our recently reported B6 vitamer concentrations in CSF of children aged eight months to 16 years (*n*=20) [Van der Ham et al (2012)]. (Table 3) Interestingly, in neocortex of 30 days old rat pups, B6 vitamer concentrations have been reported to be PMP>PM>PLP>PL, with PMP being approximately 64% of total vitamin B6. PN was not detectable and PNP and PA were not measured. [Groziak and Kirksey (1987)]

Surprisingly, concentrations of not only PL(P) but also PA and PM were much higher in newborn infants than in our group of older children [Van der Ham et al (2012)]. PLP concentrations were also higher than those reported in other studies [Footitt et al (2011)] [Ormazabal et al (2008)], in which CSF of children in different age categories was analyzed. In our subgroup of newborn infants aged ≥42 weeks, B6 vitamer concentrations were still high, but showed a strong decrease towards our recently reported concentrations in older children [Van der Ham et al (2012)]. (Table 3)

In this study, CSF was withdrawn from a subcutaneous intraventricular reservoir, whereas in our previous study we used CSF obtained by lumbar puncture. Because concentrations are not affected by a rostrocaudal gradient [Van der Ham et al (2012)], B6 vitamers can be measured in a random CSF sample. Intraventricular CSF withdrawal can therefore not explain the observed differences in B6 vitamer concentrations between newborn infants and older children. This conclusion is further strengthened by the observation that concentrations in newborns aged ≥42 weeks (CSF withdrawn from subcutaneous intraventricular reservoir)

Table 3 B6 vitamer concentrations in CSF of children in different age categories as described by Van der Ham et al (2012), Footitt et al (2011) and Ormazabal et al (2008).

Age group	Range of B6 vitamer concentration (nM) in CSF	
<30 days	PLP:	26-69 (*n*=7) 32-78 (*n*=7)
1-12 months	PLP:	14-92 (*n*=37) 24-87 (*n*=16)
1-2 years	PLP:	11-64 (*n*=28) 14-59 (*n*=18)
3-51 years 3-19 years	PLP:	10-37 (*n*=49) 11-40 (*n*=39)
8 months - 16 years	PL: PM: PN: PA: PLP: PMP:	14.8-42.5 0.1-0.5 <0.03 0.09-4.1 8.8-42.0 <5.4 (*n*=20)

strongly decrease to reach concentrations in older children (CSF obtained by lumbar puncture [Van der Ham et al (2012)]).

We investigated whether the amounts of cells and protein in CSF influenced B6 vitamer concentrations. Erythrocyte, leukocyte and protein concentrations in 67 out of 69 CSF samples were increased (>100/mL, >10/mL and >0.40 mg/mL [Van der Ham et al (2012)], respectively). However, no correlation of erythrocyte, leukocyte and protein content of CSF with B6 vitamer concentrations was observed (Spearman's rho <0.500 and/or p>0.05), making a confounding effect of cell and protein contamination on B6 vitamer concentrations in CSF of newborn infants unlikely.

Nutrition is another possible confounding factor. Breast milk is known to contain primarily pyridoxal [Ooylan et al (2002)] whereas breast milk fortifier and (preterm) infant formula contain pyridoxine (40-120 µg/100 mL [Vermeer (2011)]). Parenteral nutrition also provides the newborn infant with pyridoxine (490 micrograms per kg body weight per day). Although the type of nutrition differed between individual newborns, B6 vitamer and especially PLP concentrations did not depend on the type of nutrition (data not shown).

The two very preterm newborns (postmenstrual age <30 weeks) both showed a different B6 vitamer profile of PL>PA>PLP>PM>PN in CSF. Concentrations of PL, PA and PM were higher compared to newborns with a postmenstrual age >30 weeks, PN was present in quantifiable

amounts and PLP was relatively low. Both these newborns received parenteral nutrition (490 micrograms pyridoxine per kg body weight per day). Only in case of newborn B there was prenatal maternal supplementation with pyridoxine (25-50 mg/day; indication: nausea of pregnancy). CSF samples were taken 8 (newborn A) and 19 (newborn B) days postnatally. The similarity between the B6 vitamer profiles in newborns A and B and the long period between birth and CSF withdrawal led us to the hypothesis that maternal supplementation is not likely to be the cause of the observed differences in B6 vitamer profiles in these very preterm newborns.

The observed higher concentrations of only the unphosphorylated B6 vitamers, including PN, in CSF of these very preterm newborns may point to an immaturity of the enzymatic system involved in vitamin B6 homeostasis at a lower postmenstrual age. This is strengthened by the fact that B6 vitamer concentrations in an additional CSF sample of newborn B, withdrawn at a postmenstrual age of 34 weeks, were consistent with values found in the subgroup of preterm newborns (postmenstrual age 30-37 weeks).

This observation is also in line with the study of Raiten et al, who found continuously low serum PLP concentrations in preterm newborn infants <30 weeks of GA receiving intravenous pyridoxine [Raiten et al (1991)]. Moreover, in preterm newborns ≤28 weeks of GA, serum PLP concentrations remained low even after supplementation with pyridoxal as well as pyridoxine. In these infants however, erythrocyte PLP concentrations increased with pyridoxine supplementation. [Raiten et al (1991)] The authors suggest that increased tissue demands or metabolic trapping of PLP and/or precursors by peripheral tissues and/or erythrocytes could underlie these observations.

Since conversion of PL into PLP is independent of the pyridox(am)ine phosphate oxidase enzyme (Figure 1), possible explanations for the failure of pyridoxal to induce a serum PLP response could be a decreased activity of pyridoxal kinase or an increased activity of the (either membrane-bound alkaline or intracellular specific) phosphatase, responsible for respectively phosphorylation of PL and hydrolysis of PLP, the latter of which is necessary for transport of vitamin B6 into and out of brain cells. Both these possibilities, which are in great part also discussed by Raiten et al, would fit our observation of higher concentrations of the unphosphorylated B6 vitamers, including PL, in the presence of relatively low PLP in CSF of very preterm newborns. However, literature is inconsistent regarding the time span of development of the enzymatic system involved in vitamin B6 homeostasis throughout gestation.

Investigation of brain homeostasis in newborn infants is extremely challenging, but clinically highly important. We were able to collect a unique set of CSF samples from preterm

and term newborns who had a subcutaneous intraventricular reservoir to lower intracranial pressure by CSF withdrawal. We used these samples to study vitamin B6 homeostasis in newborn infants, as differences between preterm and term newborns have been suggested. Interpretation of our findings requires some caution since CSF could only be obtained from newborns with a post-hemorrhagic ventricular dilatation or congenital hydrocephaly. However, this is considered for the whole group studied and our approach provides the best possible reflection of brain homeostasis in healthy newborn infants.

CONCLUSION

Our results indicate that vitamin B6 homeostasis in brain differs substantially between preterm and term newborn infants. Vitamin B6 vitamer reference values for older children are inappropriate for application in newborns and age-specific B6 vitamer reference values, taking postmenstrual age into account, are indispensable for diagnosis and treatment of epilepsy and vitamin B6 deficiency in newborn infants.

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CHAPTER 4

Vitamin B6 vitamers in human plasma and cerebrospinal fluid

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ABSTRACT

Vitamin B6 comprises a group of six compounds (vitamers) that are metabolically interrelated. Insight in human vitamin B6 metabolism and transport may be gained by studying concentrations of all B6 vitamers in human plasma as well as cerebrospinal fluid (CSF).

B6 vitamer concentrations in plasma and CSF of 70 children (aged 1-18 years) and 533 adults (aged 18-63 years) were determined by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Ratios and correlations in and between plasma and CSF were studied as well as possibly influencing factors.

The B6 vitamer composition of plasma (pyridoxal phosphate (PLP) > pyridoxic acid (PA) > pyridoxal (PL)) differed from that of CSF (PL>PLP>PA> pyridoxamine (PM)). Strong correlations were observed between PLP and PL in plasma, between PA and PL in CSF and for PL between CSF and plasma. In addition to age, sex influenced concentrations of PL and PLP in plasma and CSF. Epilepsy was not of influence, but treatment with anti-epileptic drugs (AEDs) lowered PL, PLP and PM in CSF.

In conclusion, we provide B6 vitamer concentrations in plasma and CSF of a large number of children and adults. The observation of strict ratios and strong correlations points to a tight regulation of B6 vitamers in and between blood and CSF. For adequate interpretation, the influence of sex, age and AED therapy should be taken into account. B6 vitamers in plasma and CSF may be very useful in clinical practice regarding diagnosis and treatment of conditions associated with altered vitamin B6 metabolism.

INTRODUCTION

Vitamin B6 (pyridoxal phosphate (PLP)) is essential for normal human brain development and functioning, given its role as a co-factor in amino acid and neurotransmitter metabolism. Specific inborn errors of metabolism result in functional vitamin B6 deficiency, for example antiquitin deficiency (OMIM #266100) [Mills et al (2006)], pyridox(am)ine-5'-phosphate oxidase (PNPO) deficiency (OMIM #610090) [Mills et al (2005)], hypophosphatasia (alkaline phosphatase (ALPL) deficiency; OMIM #241500) [Waymire et al (1995)] [Whyte et al (1988)] and hyperprolinaemia type II (pyrroline-5-carboxylate dehydrogenase deficiency; OMIM #239510) [Walker et al (2000)]. Patients present with convulsions and, frequently, developmental delay [Stockler et al (2011)]. Diagnosing such disorders can be difficult, since biochemical abnormalities are not always present. Although treatment with vitamin B6 (pyridoxine (PN) or PLP) is often successful in reducing convulsions, developmental delay still occurs [Stockler et al (2011)] [Bok et al (2012)].

Whereas the enzymes known to be involved in human vitamin B6 metabolism have been characterized at genetic and protein levels, knowledge on transport of vitamin B6 is limited. To deepen the understanding of human vitamin B6 metabolism and transport, it is essential to study concentrations of all B6 vitamers, in plasma as well as cerebrospinal fluid (CSF) [Mills et al (2005)] [Footitt et al (2013)]. This may not only add to our understanding of the pathophysiology of disorders associated with functional vitamin B6 deficiency, but also provide us with new approaches towards diagnosis and optimization of treatment.

In literature, concentrations of the B6 vitamers PN, pyridoxamine (PM), pyridoxamine phosphate (PMP), pyridoxal (PL), PLP and the degradation product of vitamin B6, pyridoxic acid (PA), have been reported for plasma. In the majority of publications [Shephard et al (1987)] [Bates et al (1999) – Clin Chim Acta] [Bates et al (1999) – Public Health Nutr] [Driskell et al (2000)] [Talwar et al (2003)] [Midttun et al (2007)] [Marszałł et al (2009)] [Midttun et al (2009)] [Footitt et al (2013)], PLP, PA and PL were reported to be the B6 vitamers abundantly present in plasma. PM, PMP and PN were absent or their concentrations in plasma varied between studies (Supplementary Table 1).

We recently reported B6 vitamer concentrations in CSF of newborn infants (*n*=69 [Albersen et al (2012)]) and children (*n*=20 [Van der Ham et al (2012)]) (Supplementary Table 2). PL was found to be the most abundant B6 vitamer, followed by PLP. PA and PM were present in low concentrations only. PMP and PN were below limits of quantification (LOQ) and pyridoxine phosphate (PNP) could not be quantified [Van der Ham et al (2012)].

Humans are dependent on dietary sources of vitamin B6, since we are unable to synthesize vitamin B6. In our diet, various B6 vitamers are present, which are converted into

PLP. In recent *in vitro* studies, we showed that the intestine plays an important role in the conversion of precursor B6 vitamers (PN and PM) into PLP and PL [Albersen et al (2013)]. Both uptake of vitamin B6 from the diet and subsequent intestinal and hepatic metabolism result in PLP being the dominant B6 vitamer in plasma. This is in contrast to CSF, where PL is most abundant. Knowledge on the transport system from blood to brain is limited and the relationship of B6 vitamer concentrations between blood and CSF is not known. For optimal comparison between plasma and CSF, both body fluids should be obtained simultaneously and from the same individual. B6 vitamer concentrations in plasma and CSF have never been studied in this way.

In order to increase insight in human vitamin B6 metabolism and transport, we determined concentrations of PN, PM, PMP, PL, PLP and PA in plasma and CSF of children and adults. In addition, we studied the relationship between B6 vitamers *in* plasma and CSF as well as *between* CSF and plasma. We additionally investigated the possible influence of subject characteristics such as sex, age and epilepsy with or without anti-epileptic drug (AED) treatment.

SUBJECTS AND METHODS

Subjects and sample collection

Children

Plasma and/or CSF were collected from 70 children (1-18 years of age) visiting the Sylvia Tóth Center (Wilhelmina Children's Hospital, University Medical Center (UMC) Utrecht, The Netherlands) for diagnostic evaluation of developmental delay, between November 2005 and January 2011. Plasma was obtained by venous sampling (4 mL in heparin tube) and subsequent immediate centrifugation (3000 rpm, 5 min). CSF was obtained by lumbar puncture (fraction IV, according to the standardized procedure described by Van der Ham et al [Van der Ham et al (2012)]). An additional set of CSF samples obtained from 35 epileptic children using one or more AEDs (1-18 years of age; Sylvia Tóth Center and other, remnant samples) were collected to study the influence of AED therapy on B6 vitamer concentrations. Parental informed consent was obtained for all samples. Approval by the Medical Ethics Committee of the UMC Utrecht was obtained.

Adults

Of 533 healthy adults (18-63 years of age), fasting plasma and/or CSF were collected at outpatient pre-operative screening facilities of different hospitals in and near Utrecht,

The Netherlands, preceding the administration of spinal anaesthesia for minor elective surgery between August 2008 and November 2011. Subject characteristics and details of sample collection have been described previously [Luykx et al (2012)] [Luykx et al (2013) – Neuropsychopharmacology] [Luykx et al (2013) – Mol Psychiatry]. In summary, subjects were included when they were of North-Western European descent (i.e. all grandparents born in The Netherlands, Belgium, France, Germany, Denmark or The United Kingdom). Subjects with a history of self-reported psychotic and/or major neurological disorders (stroke, brain tumours and neurodegenerative disease) were excluded.

Sample storage

After withdrawal, plasma and CSF samples were stored at -80°C and protected from light until further analysis.

To study the influence of temperature on B6 vitamer concentrations in CSF, aliquots stored at -20°C were compared to aliquots stored at -80°C (reference temperature; *n*=62).

Determination of vitamin B6 vitamer concentrations

Concentrations of PN, PM, PMP, PL, PLP as well as PA were determined in plasma and CSF (nmol/L) by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) according to the method of Van der Ham et al [Van der Ham et al (2012)]. This method, developed for analysis of CSF, was adapted to measure B6 vitamers in plasma. See Supplementary Methods for details regarding the determination of B6 vitamer concentrations in plasma by UPLC-MS/MS.

Statistical analysis

SPSS 20.0 (IBM Corporation, Somers, NY) was used for statistical analysis. Because none of the B6 vitamers in plasma and CSF, nor their unstandardized residuals, showed a normal distribution, nonparametric tests (Mann-Whitney U for comparison of two groups and Mann-Whitney U with Bonferroni correction for comparison of more than two groups) were applied to study differences in median B6 vitamer concentrations. Spearman's rho (ρ) was used to describe correlations.

RESULTS

In plasma and CSF of children and adults, PL, PLP and PA were present. PM was only present in quantifiable amounts in CSF, because plasma concentrations of PM were below the limit of quantification (LOQ). PMP was <LOQ in CSF [Van der Ham et al (2012)], whereas in plasma it was not detectable due to instability. PN was <LOQ in both CSF and plasma (Supplementary Table 3). Therefore, only concentrations of PM (in CSF), PL, PLP and PA (in plasma and CSF) were studied in more detail.

A total of three children and 10 adults with one or more extremely low or high B6 vitamer concentrations in plasma and/or CSF were excluded. As a result, B6 vitamer concentrations in plasma and/or CSF of 67 children (plasma *n*=60, CSF *n*=57 and both *n*=50) and 523 adults (plasma *n*=502, CSF *n*=424 and both *n*=404) remained for further analysis.

In two children, plasma and CSF samples were not drawn simultaneously, but 21 and 22 days apart. Relative B6 vitamer concentrations did not differ from those in the other 65 children.

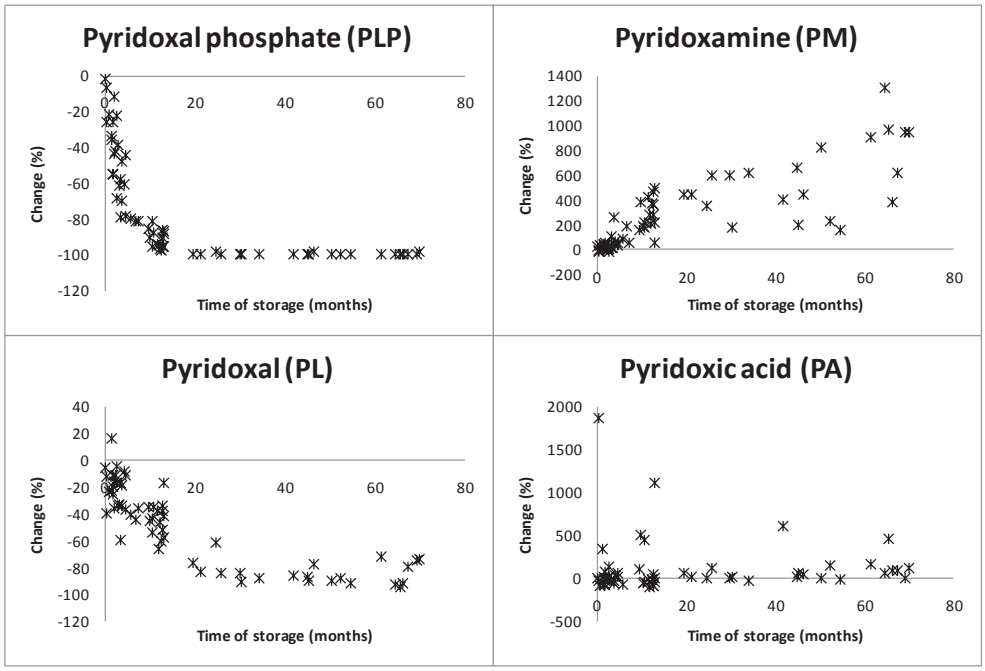


Figure 1 Change (%) in B6 vitamer concentrations in CSF with time (months), when stored at -20°C compared to -80°C (reference temperature).

Influence of storage temperature

PLP, PL, PM and PA in CSF were not stable during storage at -20°C when compared to storage at -80°C (Figure 1). Concentrations of PLP and PL decreased with time, and became undetectable after 10 and 20 months, respectively. On the contrary, concentrations of PM increased up to 500% in 15 months. For PA, no time-related trend was observed, since concentrations randomly changed from -100% to almost +2000%.

Influence of epilepsy and treatment with AEDs

The influence of epilepsy and treatment with AEDs was studied in children only, since none of the adults had epilepsy and/or was treated with AEDs. Of all 67 children, 37 did not have epilepsy nor used any AEDs, 11 did have epilepsy for which they did not use any AEDs and 19 had epilepsy and used one or more AEDs.

B6 vitamer concentrations in plasma and concentrations of PM, PL and PA in CSF did not differ between these groups. Median PLP concentrations were lower in CSF of children using one or more AEDs (14.8 nmol/L) than in CSF of children with untreated epilepsy (19.4 nmol/L, p=0.016).

To validate our finding that concentrations of PLP in CSF are decreased by the use of AEDs, B6 vitamer concentrations in an additional set of CSF samples obtained from epileptic children using one or more AEDs (1-18 years of age; *n*=35) were compared with B6 vitamer concentrations in CSF of children with and without epilepsy not using any AEDs, since there was no influence of epilepsy itself (total sample *n*=41).

Children with AED therapy showed lower median concentrations of PL (21.7 versus 28.2 nmol/L; p=6.9E-04) and PLP (15.7 versus 18.2 nmol/L; p=0.013) in CSF as well as lower median concentrations of PM (0.3 versus 0.5 nmol/L; p=6.4E-04) compared to children not using any AEDs. Median concentrations of PA did not differ significantly from those in CSF of children not using any AEDs (1.2 versus 0.9 nmol/L; p=0.098).

In nine children of the total group of 51 children with AED therapy, concentrations of PL in CSF were below the lower limit observed in children not using any AEDs (<16.1 nmol/L), whereas PLP in CSF was decreased in seven children. Three of these children showed decreased concentrations of both PL and PLP in CSF. Concentrations of PM in CSF of 13 children with AED therapy were below the lower limit observed in children not using any AEDs (<0.3 nmol/L). Two of these children also showed a decreased concentration of PL in CSF. In addition, one child with a decreased concentration of PL in CSF also showed a decreased concentration of PLP in plasma (<20.5 nmol/L).

Concentrations of PL, PLP and PM in CSF did not differ between subgroups of AED therapy (valproate monotherapy (*n*=16), valproate combined with at least one other AED (*n*=15) and monotherapies and/or combinations of other AEDs (*n*=20); data not shown).

Since AEDs influenced concentrations of PL, PLP and PM in CSF and one child displayed a decreased concentration of PLP in plasma, the group of epileptic children with AED therapy (*n*=19) was excluded from the original dataset (remaining children *n*=48; plasma *n*=42, CSF *n*=41 and both *n*=35).

B6 vitamer concentrations in plasma and CSF

Table 1 displays median concentrations of the different B6 vitamers in plasma and CSF (nmol/L) of children and adults, with their respective ranges. See also Figure 2. The most abundant B6 vitamer in plasma was PLP, whereas in CSF, the concentration of PL was the highest.

Table 1 Concentrations of PM, PL, PLP and PA (nmol/L) in plasma and/or CSF of children (1-18 years; *n*=48) and adults (18-63 years; *n*=523).

B6 vitamer concentration (nmol/L)	Body fluid	Age (years)	Median	Range
PM	***CSF***	***1-18***	0.5	0.3 - 0.9
		>18	0.4	0.0 - 1.2
PL[a]	***Plasma***	***1-18***	21.1	8.8 - 58.7
		>18	10.5	3.0 - 56.2
	CSF	***1-18***	28.2	16.1 - 55.7
		>18	30.0	13.5 - 78.5
PLP[a]	***Plasma***	***1-18***	33.9	20.5 - 151
		>18	55.9	10.2 - 335
	CSF	***1-18***	18.2	11.0 - 33.7
		>18	16.1	5.3 - 49.2
PA	***Plasma***	***1-18***	24.7	8.8 - 104
		>18	23.6	2.7 - 243
	CSF	***1-18***	0.9	0.0 - 3.1
		>18	1.2	0.0 - 8.7

[a] Sex-related differences in concentrations of PL and PLP in plasma and CSF are given in Supplementary Table 4.

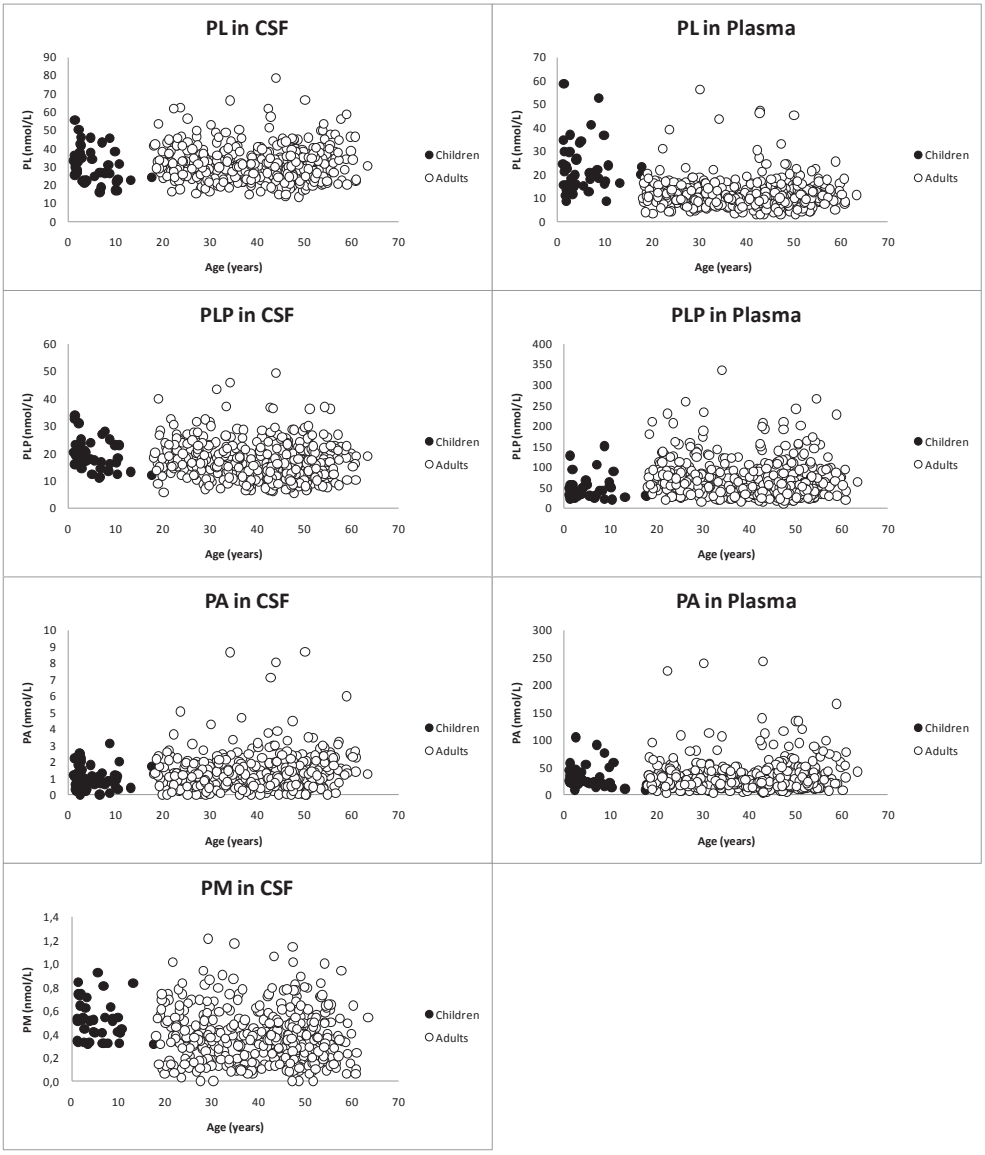


Figure 2 Concentrations of PM, PL, PLP and PA (nmol/L) in plasma and/or CSF of children (1-18 years) and adults (18-63 years).

Influence of sex

Median concentrations of PL in CSF were lower in boys than in girls (24.3 nmol/L versus 33.1 nmol/L, respectively; p=0.014, Supplementary Table 4). Concentrations of the other B6 vitamers in plasma and CSF did not differ between boys and girls (data not shown).

In plasma and CSF of adults, concentrations of both PL and PLP were influenced by sex. The median concentration of PL in CSF was lower in men (29.5 versus 32.1 nmol/L, p=0.022), whereas the median concentration of PL in plasma was higher in men (11.0 versus 9.2 nmol/L, p=3.6E-05; Supplementary Table 4). Median concentrations of PLP in both plasma and CSF were higher in men than in women (60.2 versus 44.0 nmol/L for plasma, p<5.0E-07; 17.0 versus 14.0 nmol/L for CSF, p=3.0E-04). Concentrations of the other B6 vitamers in plasma and CSF did not differ between men and women (data not shown).

Influence of age

Concentrations of PL and PLP in CSF of children only marginally decreased from one to 18 years of age (ρ = -0.379, p=0.015 for PL; ρ = -0.338, p=0.031 for PLP) and the lower concentration limits did not differ. B6 vitamer concentrations in plasma and CSF of adults did not correlate with age (data not shown).

B6 vitamer ratios and correlations *in* and *between* plasma and CSF

Table 2 shows ratios as well as correlations between PM, PL, PLP and PA in plasma and/or CSF. In plasma, the strongest correlation was observed between PLP and PL (Figure 3A). In CSF, especially concentrations of PA and PL were correlated in both age categories.

In Table 3, ratios and correlations are shown for PL, PLP and PA between CSF and plasma. The strongest correlation was for PL, in both children and adults (Figure 3B).

Table 2 Ratios between PM, PL, PLP and PA in plasma and/or CSF of children and adults.

B6 vitamer ratio	Body fluid	Age (years)	Median	Range	Correlation (rho (ρ))
PL:PM	***CSF***	***1-18***	70.3	27.7 - 154	0.090
		>18	84.4	16.6 - 774[b]	0.147*
PLP:PL	***Plasma***	***1-18***	1.9	1.0 - 4.2	0.622**
		>18	5.4	1.1 - 36.8	0.564**
	CSF	***1-18***	0.6	0.4 - 1.4	0.539**
		>18	0.5	0.2 - 1.8	0.265*
PLP:PM	***CSF***	***1-18***	40.3	16.5 - 108	-0.042
		>18	49.5	7.8 - 331[b]	0.014
PA:PL	***Plasma***	***1-18***	1.3	0.4 - 3.7	0.514**
		>18	2.4	0.3 - 15.2	0.395*
	CSF	***1-18***	0.03	0.00 - 0.07	0.565**
		>18	0.04	0.00 - 0.13	0.534**
PA:PLP	***Plasma***	***1-18***	0.7	0.2 - 2.6	0.614**
		>18	0.4	0.03 - 2.5	0.414*
	CSF	***1-18***	0.05	0.00 - 0.14	0.279
		>18	0.07	0.00 - 0.38	0.198*
PA:PM	***CSF***	***1-18***	2.0	0.0 - 7.3	-0.103
		>18	3.2	0.0 - 44.0[b]	0.171*

* = significant at the p<5.0E-03 level

** = significant at the p<5.0E-03 level and ρ > 0.500

[b] For PL:PM, PLP:PM and PA:PM in CSF, *n*=6 samples were excluded, since PM concentrations were 0 nmol/L and therefore ratio calculations were not possible.

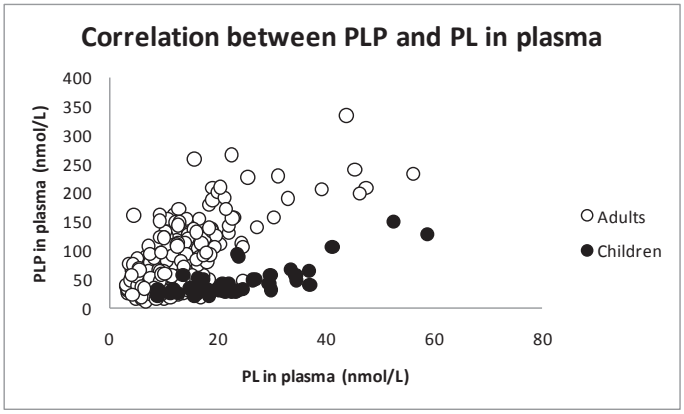
Table 3 Ratios of PL, PLP and PA between CSF and plasma of children and adults.

B6 vitamer in CSF:plasma	Age (years)	Median	Range	Correlation (rho (ρ))
PL	***1-18***	1.3	0.9 - 2.4	0.806**
	>18	2.9	0.9 - 10.6	0.467*
PLP	***1-18***	0.5	0.2 - 0.8	0.524**
	>18	0.3	0.1 - 0.9	0.629**
PA	***1-18***	0.03	0.00 - 0.28	0.226
	>18	0.04	0.00 - 0.35	0.485*

* = significant at the p<5.0E-03 level

** = significant at the p<5.0E-03 level and ρ > 0.500

A



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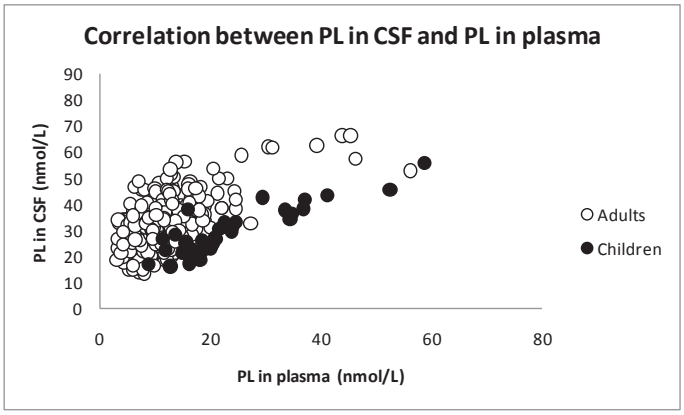

Figure 3

A. Correlation (Spearman's rho, p-value) between PLP and PL in plasma of children (ρ = 0.622, p<1.15E-05) and adults (ρ = 0.564, p<5.0E-07).

B. Correlation (Spearman's rho, p-value) between PL in CSF and PL in plasma of children (ρ = 0.806, p<5.0E-07) and adults (ρ = 0.467, p<5.0E-07).

DISCUSSION

In this study, we show B6 vitamer concentrations in plasma and CSF of a large number of children and adults. For determination of B6 vitamer concentrations we developed and validated a sensitive and accurate method [Van der Ham et al (2012)]. Because of the unique nature of our study populations, we were able to compare B6 vitamer concentrations in both body fluids and obtain further insight in metabolism and transport of vitamin B6. Since clinical information was available of all subjects, we were able to study the effect of sex, age, epilepsy and AED therapy.

To date, we do not know the physiological importance of each B6 vitamer nor the best way to evaluate a person's vitamin B6 status. By simultaneously studying B6 vitamer concentrations in plasma and CSF, this study provides thorough insight in B6 vitamer concentrations in plasma and CSF and their interrelationship.

B6 vitamers in CSF are not affected by a rostrocaudal concentration gradient [Van der Ham et al (2012)], and can therefore be measured in any CSF fraction. As shown in this study and as routinely done in our laboratory, CSF must be stored at -80°C immediately after withdrawal and until further analysis. The observed decrease of PL and PLP together with an increase of PM and changes in PA implicate that these B6 vitamers are unstable in CSF when stored at -20°C. The same holds true for plasma, since stability of B6 vitamers in plasma stored at -80°C has been described [Bates et al (1999) – Clin Chim Acta] [Marszałł et al (2009)].

B6 vitamers in human plasma and CSF

We show that the B6 vitamer composition of plasma (PLP>PA>PL) differs from that of CSF (PL>PLP>PA>PM). PM was not detectable in plasma, as was also previously reported [Marszałł et al (2009)] [Midttun et al (2009)]. Likewise, PN was not present in plasma nor in CSF, unless subjects were supplemented with vitamin B6 [Van der Ham et al (2012)].

By determining concentrations of PM, PL, PLP and PA in plasma and/or CSF, we provide reference values in both children and adults. Although we used plasma and CSF of children visiting the Sylvia Tóth Center for diagnostic evaluation of developmental delay, this approach provides the best possible reflection of healthy vitamin B6 homeostasis. Decreased or elevated B6 vitamer concentrations can be used in the diagnosis of known functional vitamin B6 deficiencies, as well as yet uncharacterized disorders of vitamin B6 metabolism and/or transport. Indeed, low concentrations of PLP [Mills et al (2005)] [Ruiz et al (2008)] [Goyal et al (2013)] and PL [Mills et al (2005)] have been found in CSF of patients

with PNPO deficiency as well as antiquitin deficiency (low PLP only [Footitt et al (2011)]). Concentrations of the other B6 vitamers were not reported, while these might be abnormal as well and may have differential diagnostic impact.

Epilepsy and treatment with AEDs

Our study demonstrates that B6 vitamer concentrations in CSF are not influenced by epilepsy. This finding is supported by the study of Footitt et al, although they only studied PLP in CSF [Footitt et al (2011)]. However, we did find lower PL and PLP as well as PM concentrations in CSF of epileptic children using one or more AEDs, compared to children not using any AEDs. This implies that children with AED therapy are at risk of a deficiency of vitamin B6, which might have adverse effects on brain development and functioning.

B6 vitamer concentrations in plasma were not influenced by epilepsy nor by AEDs. However, one child with AED therapy showed a decreased concentration of PL in CSF as well as a decreased concentration of PLP in plasma. In literature, several AEDs have been associated with decreased concentrations of PLP in plasma, for adults [Schwaninger et al (1999)] [Apeland et al (2002)] [Apeland et al (2003)] [Apeland et al (2006)] as well as children [Verrotti et al (2000)] [Vilaseca et al (2000)]. In addition, Apeland et al reported lower plasma PA concentrations in adults using one or more AEDs [Apeland et al (2003)].

Sex and age

Concentrations of PL and PLP in plasma and CSF of adults and concentrations of PL in CSF of children were influenced by sex. Associations between B6 vitamer concentrations and sex have been reported before. Both Gonzalez-Gross et al and Kerr et al observed higher plasma PLP concentrations in boys compared to girls, although concentration ranges were comparable [Gonzalez-Gross et al (2012)] [Kerr et al (2009)].

For age, we found a negative correlation with CSF concentrations of PL and PLP in children. A negative correlation between CSF concentrations of PLP and age in children has been previously reported [Footitt et al (2011)] [Ormazabal et al (2008)].

Vitamin B6 metabolism

The strong correlations between PLP and PL in plasma and between PA and PL in CSF suggest that concentrations of these B6 vitamers are tightly regulated (Supplementary Table 5). Disturbances of B6 vitamer ratios in plasma and/or CSF may therefore indicate possible deficiencies of the enzymes involved in vitamin B6 metabolism: pyridoxal kinase (which

phosphorylates PL into PLP) and pyridoxal phosphatase (which hydrolyzes PLP into PL [Jang et al (2003)]) as well as pyridoxal oxidase, which is involved in the degradation of PL into PA [Merrill et al (1984)]. It is therefore relevant to determine concentrations of all B6 vitamers when investigating possible vitamin B6 related disease.

Interestingly, the correlation between PLP and PL in plasma differed between adults and children. Plasma concentrations of PLP were relatively higher in adults, whereas plasma concentrations of PL were relatively higher in children. Although both groups cannot be strictly compared to each other, because adults were healthy and fasting and children were developmentally delayed, one might hypothesize that age-related changes in the activity of alkaline phosphatase (ALPL; OMIM *171760), which hydrolyzes PLP into PL, might underlie our observation, since it is known that ALPL activities in plasma are higher in children than in adults [Fleisher et al (1977)] [Schiele et al (1983)]. The relationship between PLP in plasma and ALPL has been shown by a genome-wide association study (GWAS), in which a single nucleotide polymorphism (SNP) in the *ALPL* gene (rs1256335 [Hazra et al (2009)]) was associated with concentrations of PLP in plasma. Subjects carrying the G allele had lower plasma PLP concentrations than subjects homozygous for the A allele [Hazra et al (2009)]. Furthermore, the inborn error of metabolism hypophosphatasia, caused by a deficiency of ALPL (OMIM #241500), is known to result in functional vitamin B6 deficiency [Waymire et al (1995)] [Whyte et al (1988)].

Vitamin B6 transport

Little is known about the mechanism by which any of the vitamers is transported from blood to brain. At a biochemical level, there is evidence for carrier-mediated transport in choroid plexus and blood brain barrier [Spector and Johanson (2007)], but a vitamin B6 transporter protein has not yet been characterized.

The strong correlation between PL in CSF and PL in plasma but primarily the observation that concentrations of PL are higher in CSF than in plasma, suggest that transport of PL takes place through an active mechanism at the blood brain barrier or choroid plexus. Disturbances of B6 vitamer ratios between CSF and plasma may therefore point towards a problem in vitamin B6 transport, in a way similar to the decreased CSF:plasma ratio of glucose that is found in GLUT1 (blood brain barrier glucose transporter) deficiency (OMIM #606777). We therefore advocate to not only analyze B6 vitamers in plasma or CSF, but in both body fluids simultaneously when investigating a functional vitamin B6 deficiency of unknown cause.

Interestingly, the correlation between PL in CSF and PL in plasma was different in adults compared to children, since CSF concentrations of PL were relatively higher in adults. One might hypothesize that transport of PL from blood to brain differs between adults and children, although both groups are not strictly comparable.

CONCLUSION

With this study, we provide thorough insight in B6 vitamer concentrations in plasma and CSF of a large number of children as well as adults. It is evident that samples should be stored at -80°C. The observation of strict ratios and strong correlations points to a tight regulation of B6 vitamers in and between blood and CSF. For adequate interpretation of B6 vitamer concentrations, the influence of sex, age and AED therapy should be taken into account. B6 vitamers in plasma and CSF may be very useful in clinical practice regarding diagnosis and treatment of conditions associated with altered vitamin B6 metabolism.

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SUPPLEMENTARY METHODS

For determination of B6 vitamer concentrations in plasma, 100µL of plasma and internal standards was used. Samples were centrifuged twice after protein precipitation with trichloroacetic acid (TCA) (5 min, 13000 rpm). Calibration curve end concentrations of PL, PLP and PA were adjusted for quantification of these B6 vitamers in plasma (160, 200 and 185 nmol/L, respectively). For quality control samples, plasma of random subjects was pooled and B6 vitamers were spiked to achieve three different concentration levels (QC1-3). QC1, QC2 and QC3 were used to study inter-assay variations (*n*=10) for the different B6 vitamers. Limits of detection (LOD) and quantification (LOQ) were determined using QC1 (*n*=10; signal-to-noise ratios (S/N) 3 and 10, respectively) (Supplementary Table 3). PMP in plasma was not detectable due to instability.

SUPPLEMENTARY TABLES

Supplementary Table 1 Vitamin B6 vitamer concentrations in human plasma, as reported in literature. Only the measured B6 vitamers and studies which included >10 subjects are presented.

Reference	Population	B6 vitamer in plasma (nmol/L)						
		PL	PM	PN	PA	PLP	PMP	PNP
Shephard et al (1987)	South-African healthy volunteers (30.8 ± 9.9 yrs; *n*=27) [mean ± SD (range)[a]]	9.0 ± 7.2 (4.2-43.7)	0.6 ± 0.6 (0.0-2.4)	0.6 ± 1.8 (0.0-5.3)	29.5 ± 20.8 (8.7-101)	50.6 ± 25.1 (12.1-111)	0.4 ± 0.8 (0.0-3.2)	
Bates et al (1999) - Clin Chim Acta	British elderly (≥ 65 yrs; *n*=297) [2.5-97.5 %]							
	Men (*n*=136)				7.9-33.6	17.9-98.8		
	Women (*n*=161)				6.4-40.5	14.4-98.7		
Bates et al (1999) - Public Health Nutrition	British young (4-18 yrs; *n*=1006) and older (≥ 65 yrs; *n*=919) people [G mean (95% CI)]							
	Young people				10.6 (8.6-12.6)	56.5 (54.5-58.5)		
	Older people				15.5 (13.5-17.5)	34.0 (32.0-36.0)		
Driskell et al (2000)	Healthy, non-supplemented Caucasian men and women (19-50 yrs; *n*=41) [mean ± SD]							
	1.Men (19-24 yrs; *n*=8)	7.8 ± 3.3	13.0 ± 4.9	4.3 ± 3.2	8.5 ± 7.2	95.0 ± 28.8	6.4 ± 8.7	
	2.Men (25-50 yrs; *n*=9)	9.3 ± 4.6	12.8 ± 8.0	5.4 ± 1.7	10.9 ± 5.9	90.0 ± 50.2	11.0 ± 12.4	
	3.Women (19-24 yrs; *n*=11)	5.8 ± 2.1	9.7 ± 10.2	3.0 ± 3.6	8.3 ± 6.0	70.1 ± 27.5	4.9 ± 7.3	
	4.Women (25-50 yrs; *n*=13)	6.2 ± 1.7	12.6 ± 8.7	2.9 ± 3.9	5.9 ± 4.1	74.1 ± 25.4	9.7 ± 19.1	
Talwar et al (2003)	British healthy subjects (31-73 yrs; *n*=126) [G mean (95% RI)]	11 (5-26)			23 (9-60)	56 (21-138)		

Midttun et al (2007)	Norwegian healthy subjects (50-64 yrs; *n*=10601) [median (5-95 %)]	10.0 (5.2-41.1)	present in 0.85%; max 465	present in 1.9%; max 2970	20.4 (10.3-110)	48.0 (18.7-152)	rarely present, close to 4 (LOQ)	rarely present, close to 0.2 (LOQ)
Marszałł et al (2009)	Polish volunteers (25-35 yrs; *n*=40) [mean ± SD] *(plasma and serum)*							
	Men (*n*=20)	12.7 ± 6.7	n.d.	n.d.	32.6 ± 17.8	51.4 ± 22.4	n.d.	
	Women (*n*=20)	17.6 ± 8.2	n.d.	n.d. (*n*=16); 6.9 ± 1.3 (*n*=4)	43.7 ± 21.2	62.7 ± 31.6		
Midttun et al (2009)	Norwegian healthy individuals (11-93 yrs; *n*=94) [median (5-95 %)]	10.0 (4.8-55.4)	<0.1 (0.0-0.0)	<0.05 (0.0-0.0)	24.3 (11.6-266)	37.2 (14.2-182)		
Footitt et al (2012)	British paediatric controls (4.3-16 yrs; *n*=24) [range]	4.6-18.1	n.d.	n.d.-0.62	16.4-139	46-321	n.d.-9.3	n.d.

[a] Concentration in nmol/L was calculated from an original concentration in ng/mL. n.d. = not detectable. G mean = geometric mean. % = percentile.

Supplementary Table 2 Vitamin B6 vitamer concentrations in human cerebrospinal fluid (CSF), as reported in literature. Only the measured B6 vitamers are presented.

Reference	Population	B6 vitamer in CSF (nmol/L)						
		PL	PM	PN	PA	PLP	PMP	PNP
Van der Ham et al (2012)	Children evaluated for developmental delay and/or movement disorder (8 mo-16.3 yrs; *n*=20) [range]	14.8-42.5	0.1-0.5	<0.03 (LOQ)	0.09-4.1	8.8-42.0	<5.4 (LOQ)	
Albersen et al (2012)	Newborn infants (*n*=67; postmenstrual age in weeks) [range (median)]							
	1. 30-37 weeks (*n*=25)	46-226 (105)	0.5-3.6 (1.4)	<0.03 (LOQ)	6.0-73 (15)	28-170 (101)	<5.4 (LOQ)	
	2. 37-42 weeks (*n*=23)	16-199 (102)	0.3-3.3 (1.0)	<0.03 (LOQ)	1.9-52 (13)	19-221 (106)	<5.4 (LOQ)	
	3. ≥42 weeks (*n*=19)	14-103 (49)	0.3-1.4 (0.7)	<0.03 (LOQ)	0.9-11 (4.7)	8.0-76 (32)	<5.4 (LOQ)	
Footitt et al (2011)	British subjects with neurological symptoms (*n*=121) [2.5 – 97.5 %]							
	1. <30 days (*n*=7)					26-69		
	2. 1-12 months (*n*=37)					14-92		
	3. 1-2 yrs (*n*=28)					11-64		
	4. 3-51 yrs (*n*=49)					10-37		
Ormazabal et al (2008)	Spanish paediatric controls with a neurological condition (*n*=80) [range]							
	1. <30 days (*n*=7)					32-78		
	2. 1-12 months (*n*=16)					24-87		
	3. 1-2 yrs (*n*=18)					14-59		
	4. 3-19 yrs (*n*=39)					11-40		
Shin (1984)	German control subjects (2 mo-68 yrs; *n*=88) [mean ± SD (range)[a]]							
	≤1 yr (*n*=26)					23.1 ± 16.2 (5.7-78.1)		
	1-68 yrs (*n*=62)					13.4 ± 9.7 (1.2-52.2)		

% = percentile. [a] Concentration in nmol/L was calculated from an original concentration in ng/mL.

Supplementary Table 3 Precision (inter-assay variation), limit of detection (LOD) and limit of quantification (LOQ) for the determination of B6 vitamer concentrations in plasma using UPLC-MS/MS.

B6 vitamer	Concentration (nmol/L)			LOD	LOQ	Inter-assay variation (%)		
	QC1	QC2	QC3			QC1	QC2	QC3
PN	11.4	22.3	43.8	0.08	0.28	6.4	6.0	4.3
PM	35.5	84.1	184	0.81	2.70	26	18	18
PL	62.7	100	154	0.86	2.86	7.9	5.9	6.1
PLP	84.6	158	213	0.96	3.19	12	9.7	6.2
PA	49.0	78.4	104	0.27	0.91	23	25	17

Supplementary Table 4 Differences in concentrations of PL and PLP (nmol/L) in plasma and/or CSF of male and female children (1-18 years; *n*=48) and/or adults (18-63 years; *n*=523).

B6 vitamer concentration (nmol/L)	Body fluid	Age (years)	Sex	Median	Range	Significance (p-value)
PL	***Plasma***	***>18***	***Male***	11.0	3.0 - 56.2	3.6E-05
			Female	9.2	3.2 - 33.0	
	CSF	***1-18***	***Male***	24.3	16.1 - 55.7	0.014
			Female	33.1	21.1 - 45.9	
		>18	***Male***	29.5	13.5 - 78.5	0.022
			Female	32.1	14.9 - 62.0	
PLP	***Plasma***	***>18***	***Male***	60.2	16.2 - 335	<5.0E-07
			Female	44.0	10.2 - 259	
	CSF	***>18***	***Male***	17.0	5.3 - 49.2	3.0E-04
			Female	14.0	6.3 - 43.2	

(boys *n*=28, girls *n*=20; men *n*=375, women *n*=148)

Supplementary Table 5 Vitamin B6 vitamer ratios and correlations in human plasma and CSF, as reported in literature.

Reference	B6 vitamer ratios and correlations in plasma	B6 vitamer ratios and correlations in CSF
Bates et al (1999) - Public Health Nutrition	Young people **PLP~PA** *r*=0.60 (p<0.0001) Older people **PLP~PA** *r*=0.56 (p<0.0001) [regression coefficient]	
Midttun et al (2007)	**PLP~PL** ρ=0.80 (p<0.001) **PL~PA** ρ=0.79 (p<0.001) **PLP~PA** ρ=0.67 (p<0.001) [Spearman's correlation]	
Vasilaki et al (2008)	**PLP:PL** 4.94 (2.53-44.67) [median ratio (range)] **PLP~PL** ρ=0.58 (p<0.001) [Spearman's correlation]	
Footitt et al (2012)	**PLP:PL** 5.2-18.6 **PL:PA** 0.1-0.7 [ratio]	
Albersen et al (2012)		**PA:PL** 30-37 weeks: 0.08-0.49 (0.17) 37-42 weeks: 0.04-0.28 (0.15) ≥42 weeks: 0.02-0.33 (0.08) **PLP:PL** 30-37 weeks: 0.35-2.51 (0.91) 37-42 weeks: 0.45-3.20 (1.02) ≥42 weeks: 0.15-1.92 (0.68) [ratio range (median)[c]] **PL~PM** ρ=0.687 (p<0.0005) **PLP~PM** ρ=0.297 (p=0.015) **PA~PM** ρ=0.627 (p<0.0005) **PLP~PL** ρ=0.631 (p<0.0005) **PA~PL** ρ=0.849 (p<0.0005) **PA~PLP** ρ=0.627 (p<0.0005) [Spearman's correlation] [c] Postmenstrual age in weeks

CHAPTER 5

Genome-wide association study of vitamin B6 vitamers in human plasma and cerebrospinal fluid

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In preparation

ABSTRACT

The active form of vitamin B6, pyridoxal phosphate (PLP), is of pivotal importance for normal brain development and functioning. To obtain insight in the genetic regulation of B6 vitamer concentrations as well as human vitamin B6 metabolism and transport, we conducted a genome-wide association study (GWAS) of the B6 vitamers pyridoxal (PL), PLP, pyridoxamine (PM) and the degradation product pyridoxic acid (PA) in plasma and cerebrospinal fluid (CSF) of healthy human subjects (*n*=381). Concentrations and ratios in and between plasma and CSF were studied for their association with single nucleotide polymorphisms (SNPs).

Genome-wide significant (p<5.0E-08) associations with SNPs at loci containing transporter and neurotransmitter receptor genes were identified. PLP in plasma was associated with a SNP 12.3 kb upstream of *SLC27A6* (fatty acid transporter; rs36155889, p=1.21E-09). For the ratio between PA and PLP in CSF, ten SNPs upstream of *HTR7* (serotonin receptor; p=1.29E-08 – 4.16E-08) were found. These observations provide us with insight in the genetic associations of B6 vitamer concentrations and ratios. Although the relationship between vitamin B6 and fatty acids and serotonin is known, a challenge remains in elucidating the exact mechanism by which the influence of the abovementioned SNPs is executed.

INTRODUCTION

Pyridoxal phosphate (PLP), the active form of vitamin B6, acts as a co-factor in numerous enzymatic reactions, amongst which reactions that catalyze the metabolism of several amino acids and neurotransmitters (dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), glycine and D-serine). In addition, PLP is required for the actions of, along with >160 other enzymes [Percudani et al (2009)], glycogen phosphorylase (glucose biosynthesis), cystathionine β-synthase (homocysteine metabolism) and aminolevulinate synthase (heme biosynthesis).

Inverse relationships of vitamin B6 with oxidative stress [Chetyrkin et al (2011)] [Keles et al (2010)] [Mooney et al (2009)], inflammation [Lotto et al (2012)] [Morris et al (2010)] [Paul et al (2013)] [Sakakeeny et al (2012)] [Ulvik et al (2012)], cardiovascular disease [Dhalla et al (2013)] and diabetes [Kiran et al (2011)] as well as cancer [Banque et al (2012)] [Chou et al (2011)] [Galluzzi et al (2012)] [Galluzzi et al (2013)] [Harris et al (2012)] [Johansson et al (2010)] [Larsson et al (2010)] [Le Marchand et al (2011)] [Zhang et al (2011)] [Zschäbitz et al (2013)] and cognitive impairment [Moorthy et al (2012)] [Riggs et al (1996)] have been reported.

Vitamin B6 cannot be synthesized by humans and must be obtained from dietary resources. In meat and milk products, mainly pyridoxamine phosphate (PMP) and PLP are present, whereas in vegetables and fruits, pyridoxine (PN) is the dominant B6 vitamer.

The different vitamers of vitamin B6: PN, pyridoxamine (PM), pyridoxal (PL) and their phosphate-esters, are interconvertible through the action of several enzymes. Transport across the cell membrane is preceded by hydrolysis of the phosphorylated vitamers by the enzyme alkaline phosphatase (ALPL) [Spector and Greenwald (1978)] [Waymire et al (1995)]. Inside the cell, (re-)phosphorylation of the absorbed B6 vitamers by pyridoxal kinase (PDXK), yields PNP, PMP and PLP. Pyridox(am)ine phosphate oxidase (PNPO) converts PNP and PMP into the active co-factor, PLP [Clayton (2006)]. Release from the cell is dependent on a vitamin B6-specific phosphatase (pyridoxal phosphatase (PDXP)) [Jang et al (2003)], which hydrolyzes intracellular PLP. (Figure 1) The resulting PL is converted into pyridoxic acid (PA), the major degradation product of vitamin B6 [Bender (2005)].

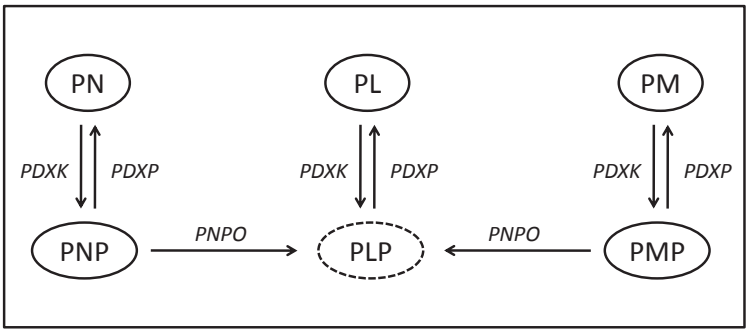

Figure 1 The different B6 vitamers and their intracellular conversions.

Vitamin B6 is of pivotal importance for normal brain development and functioning. Over the past years, interest in vitamin B6 has increased, since inborn errors of metabolism resulting in functional vitamin B6 deficiency have been identified. To date, we do not know the physiological importance of each B6 vitamer nor the best way to diagnose and treat functional vitamin B6 deficiency.

From our previous studies [Van der Ham et al (2012)] [Albersen et al (2012)] [Albersen et al (submitted)] we know that the B6 vitamer composition of human plasma differs from that of human cerebrospinal fluid (CSF). Possible mechanisms explaining this difference are (a combination of) metabolism, transport and/or genetic regulation.

Genome-wide association studies (GWAS) have not been published for vitamin B6 in CSF. For B6 vitamer (PLP) concentrations in plasma, GWAS have only scarcely been performed [Hazra et al (2009)] [Tanaka et al (2009)] (Table 1) and only a few genome-wide significant associations have been found. Single nucleotide polymorphisms (SNPs) in the *ALPL* gene (rs1256335; [Hazra et al (2009)]) and SNPs in the *NBPF3* (neuroblastoma breakpoint family, member 3) gene (rs4654748; [Hazra et al (2009)] [Tanaka et al (2009)]) were associated with the plasma concentration of PLP.

Table 1 Published genome-wide significant associations for plasma vitamin B6 (PLP).

SNP	Chr	Allele	Locus	Beta	Significance (p-value)	Body fluid & B6 vitamer	Methods	Reference
rs1256335	1	G/A	*ALPL*	-0.14 log	1.40E-15	Plasma PLP	Meta-analysis of three GWAS (*n*=4763)	[Hazra et al (2009)]
rs4654748		C/T	*NBPF3*	-0.10 log	4.30E-11			
rs4654748	1	C/T	*NBPF3*	-1.45 ng/mL	8.30E-18	Plasma vitamin B6	Meta-analysis of two GWAS (*n*=1864)	[Tanaka et al (2009)]

To study the genetic regulation of B6 vitamer concentrations, we conducted a GWAS of B6 vitamers (PL, PLP, PM) and PA in plasma and CSF of healthy human subjects (*n*=381). To obtain insight in vitamin B6 metabolism and transport, genetic regulation of ratios between B6 vitamers *in* plasma and CSF as well as ratios of B6 vitamers *between* CSF and plasma were studied. To answer the question which of the enzymes involved in vitamin B6 metabolism is or are dominant in determining B6 vitamer concentrations, SNPs in genes known to be involved in vitamin B6 metabolism (*ALPL*, *PDXK*, *PNPO* and *PDXP*) were studied for their association with B6 vitamers (PL, PLP, PM) and PA.

SUBJECTS AND METHODS

Subjects and sample collection

Subject characteristics and collection of samples have been described in detail by Luykx et al [Luykx et al (2012)] [Luykx et al (2013)]. In summary, plasma and/or CSF were collected of 533 healthy subjects undergoing spinal anesthesia for minor elective surgery between August 2008 and November 2011. Subjects were recruited at outpatient pre-operative screening facilities of different hospitals in and near Utrecht, The Netherlands. Subjects were 18-63 years of age and of North-Western European descent (i.e. all grandparents born in The Netherlands, Belgium, France, Germany, Denmark or The United Kingdom). Subjects with psychotic and/or major neurological disorders (stroke, brain tumour, neurodegenerative disease) were excluded from participation. Most of the subjects underwent knee arthroscopy and do reflect the general population with respect to comorbidities [Hetsroni et al (2011)]. The study was approved by the ethics committee of the University Medical Center (UMC) Utrecht and by all local ethics committees. The participants provided written informed consent. After withdrawal, plasma and CSF were stored at -80°C until further analysis.

Determination of vitamin B6 vitamer concentrations

Concentrations of the B6 vitamers PL, PLP, PM, PMP and PN, as well as the concentration of PA, were determined in plasma and CSF (nmol/L) by ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) [Van der Ham et al (2012)]. After exclusion of subjects with outlier B6 vitamer concentrations in plasma and/or CSF (*n*=10) [Albersen et al (submitted)], B6 vitamer concentrations in plasma and/or CSF of 523 subjects remained for genome-wide association analyses.

Phenotyping

As described previously [Van der Ham et al (2012)] [Albersen et al (2012)] [Albersen et al (submitted)], plasma and CSF concentrations of PMP and PN, and the plasma concentration of PM, are in general below limits of quantification. Since this also applied to our dataset, only PL, PLP and PA concentrations in plasma and CSF, as well as the concentration of PM in CSF, were included in the association analyses. Thus, no information on the genetic associations of the undetectable B6 vitamers was obtained in this study.

To obtain insight in not only the genetic regulation of B6 vitamer concentrations, but also in vitamin B6 metabolism and transport, concentrations of B6 vitamers as well as ratios *in* and *between* plasma and CSF were studied.

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, Somers, NY). Since none of the B6 vitamer concentrations and ratios, nor their unstandardized residuals, showed a normal distribution, B6 vitamer concentrations and ratios were log-transformed.

Genotyping, imputation and quality control procedures

From 381 out of 523 subjects of whom we determined B6 vitamer concentrations in plasma and/or CSF, whole-genome SNP data were available (Illumina HumanOmniExpress Beadchip (733 202 SNPs), UCLA Neuroscience Genomic Core facility [Luykx et al (2013)]). Collection and genetic imputation of these data have been described in detail by Luykx et al [Luykx et al (2013)].

After quality control procedures [Luykx et al (2013)], in which we applied a Hardy Weinberg Equilibrium (HWE) p-value > 1.0E-3 and a Minor Allele Frequency (MAF) ≥ 0.05, a total of 5 732 091 SNPs remained for association analyses, which were performed using Plink v1.07 [Purcell et al (2007)]. Since genomic inflation factors ranged between 0.97 and 1.03 (mean chi-squared) for all tested B6 vitamer concentrations and ratios, population stratification was not likely.

We applied a sex- and age-adjusted additive linear model (*n*=381 non-missing values) [Mittelstrass et al (2011)] [Slupsky et al (2007)] to detect SNPs relevant to B6 vitamer concentrations and ratios in and between plasma and CSF of healthy adult subjects. The threshold for genome-wide significant association was set to the generally accepted p-value < 5.0E-08 [Barsh et al (2012)] whereas the threshold for suggestively significant association was set to p < 1.0E-06.

Human Genome 19 (UCSC Genome Browser) was used for SNP annotation in SNP Nexus (http://www.snp-nexus.org/). QQ- and Manhattan plots were generated with R (http://www.r-project.org) using the GettingGeneticsDone website (http://GettingGeneticsDone.blogspot.com/) and regional association plots were created using LocusZoom (http://statgen.sph.umich.edu/locuszoom/).

Untransformed B6 vitamer concentrations and ratios were used to study the relationship between phenotype (B6 vitamer concentration or ratio) and genotype (homozygosity for the minor or major allele, or heterozygosity for both alleles) of genome-wide significant SNPs.

SNPs in genes known to be involved in vitamin B6 metabolism (*ALPL*, *PDXK*, *PNPO* and *PDXP*) and SNPs in the *ALDH7A1* gene, encoding antiquitin, were also studied for their association with B6 vitamer (PL, PLP, PM) and PA concentrations and ratios in and between plasma and CSF.

RESULTS

Descriptive statistics

Table 2 shows characteristics of the 381 genotyped subjects and their PL, PLP, PM and PA concentrations and ratios in and between plasma and CSF (*n*=367 for plasma, *n*=303 for CSF and *n*=290 for both).

Table 2 Characteristics of the 381 genotyped subjects and their B6 vitamer (PL, PLP, PM) and PA concentrations (nmol/L) and ratios in and between plasma and CSF.

		Number	Median	Range
Gender		104 (female) 277 (male)	n.a.	n.a.
Age (years)		381	42	18 - 63
PL	***Plasma***	367	11.0	3.0 - 56.2
	CSF	303	29.8	13.9 - 66.4
PLP	***Plasma***	367	57.6	10.2 - 335
	CSF	303	15.8	5.3 - 45.8
PM	***CSF***	303	0.4	0.0 - 1.2
PA	***Plasma***	367	24.6	2.7 - 243
	CSF	303	1.2	0.0 - 8.7
PL:PM	***CSF***	297[a]	79.7	16.6 - 567
PLP:PL	***Plasma***	367	5.4	1.5 - 36.8
	CSF	303	0.5	0.2 - 1.8
PLP:PM	***CSF***	297[a]	47.9	7.8 - 307
PA:PL	***Plasma***	367	2.4	0.3 - 15.2
	CSF	303	0.04	0.00 - 0.13
PA:PLP	***Plasma***	367	0.44	0.03 - 2.49
	CSF	303	0.07	0.00 - 0.38
PA:PM	***CSF***	297[a]	3.2	0.0 - 26.8
PL *in CSF:plasma*		290	2.8	0.9 - 10.6
PLP *in CSF:plasma*		290	0.3	0.1 - 0.9
PA *in CSF:plasma*		290	0.05	0.00 - 0.35

[a] For PL:PM, PLP:PM and PA:PM in CSF, *n*=6 samples were excluded, since PM concentrations were 0 nmol/L and ratio calculations were therefore not possible.

Genome-wide significant associations

B6 vitamer concentrations and ratios in plasma

In plasma, the only genome-wide significant association was found for the concentration of PLP (Table 3). One SNP on chromosome 5, 12.3 kb upstream of *SLC27A6* (solute carrier family 27, member 6), showed a genome-wide significant ($p < 5.0E-08$) association with the plasma PLP concentration (rs36155889, beta = 0.21 log, $p = 1.21E-09$; Figure 2A; Supplementary Figure 1A). In plasma of subjects heterozygous for both alleles (*n*=46), the median PLP concentration was >70% higher than in plasma of subjects homozygous for the major allele of this SNP (*n*=320, $p < 5.00E-07$, Mann-Whitney U test; Figure 3A). No genome-wide significant associations were found for concentrations of and ratios between the other B6 vitamers in plasma.

Table 3 Genome-wide significant (p < 5.0E-08) associations for B6 vitamer (PL, PLP, PM) and PA concentrations and ratios in and between plasma and CSF. Only the most significant association for each phenotype is given.

Phenotype	SNP	Chr	Position (bp)	Allele	MAF	*Locus* (relative position)	OMIM	Number	Beta	Significance (p-value)
PLP in plasma	rs36155889	5	128288867	G/A	0.07	*SLC27A6* (12.3 kb upstream)	*604196	367	0.21 log	1.21E-09
PL:PM in CSF	rs9330057	9	67051352	A/C	0.07	*AQP7P4* (2.92 kb downstream)	-	280	-0.27 log	1.56E-08
PLP:PM in CSF						*LOC286297* (19.3 kb downstream (c))	-		-0.30 log	4.22E-08
						AQP7P1 (219 kb upstream (c))	-			
PA:PLP in CSF	rs61392427	10	92105630	A/G	0.06	*SNRPD2P1* (367 kb downstream)	-	303	0.003 log	1.29E-08
						HTR7 (395 kb upstream (c))	*182137			
PA:PM in CSF	rs12150561	17	42246196	C/T	0.06	*C17orf53* (6.35 kb downstream)	-	297	0.10 log	3.38E-09
						ASB16 (1.88 kb upstream)	*615056			
PA in CSF:Plasma	rs10161007	12	77205071	C/G	0.08	*ZDHHC17* (intronic)	*607799	290	0.002 log	4.96E-08

SNP = single nucleotide polymorphism, Chr = chromosome, bp = base pair, MAF = minor allele frequency, (c) = complementary

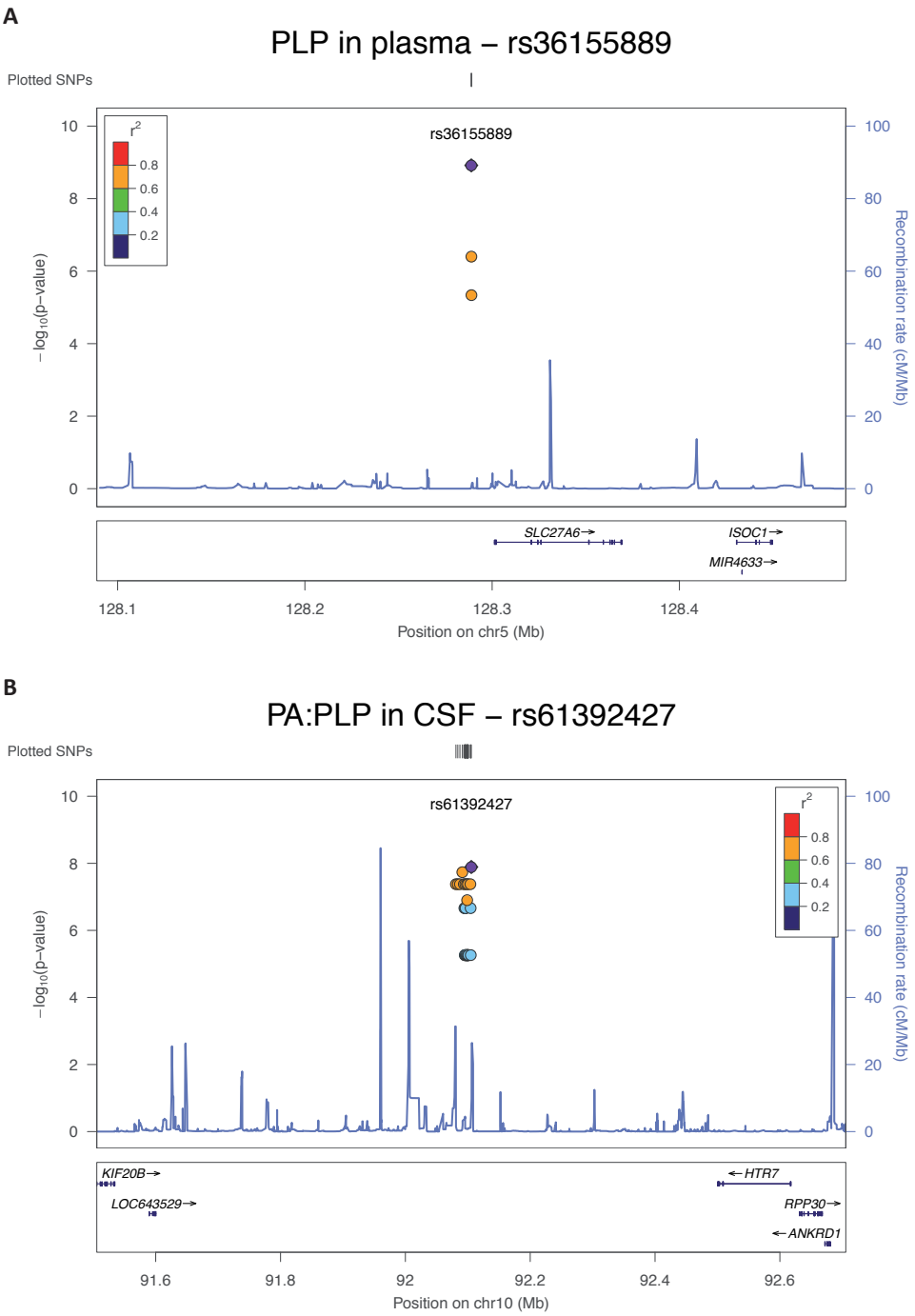

Figure 2 Regional association plots for rs36155889 and rs61392427, which showed genome-wide significant (p < 5.0E-08) associations with PLP in plasma (A) and with the ratio between PA and PLP in CSF (B), respectively (significance cut-off in plot is p < 1.0E-03).

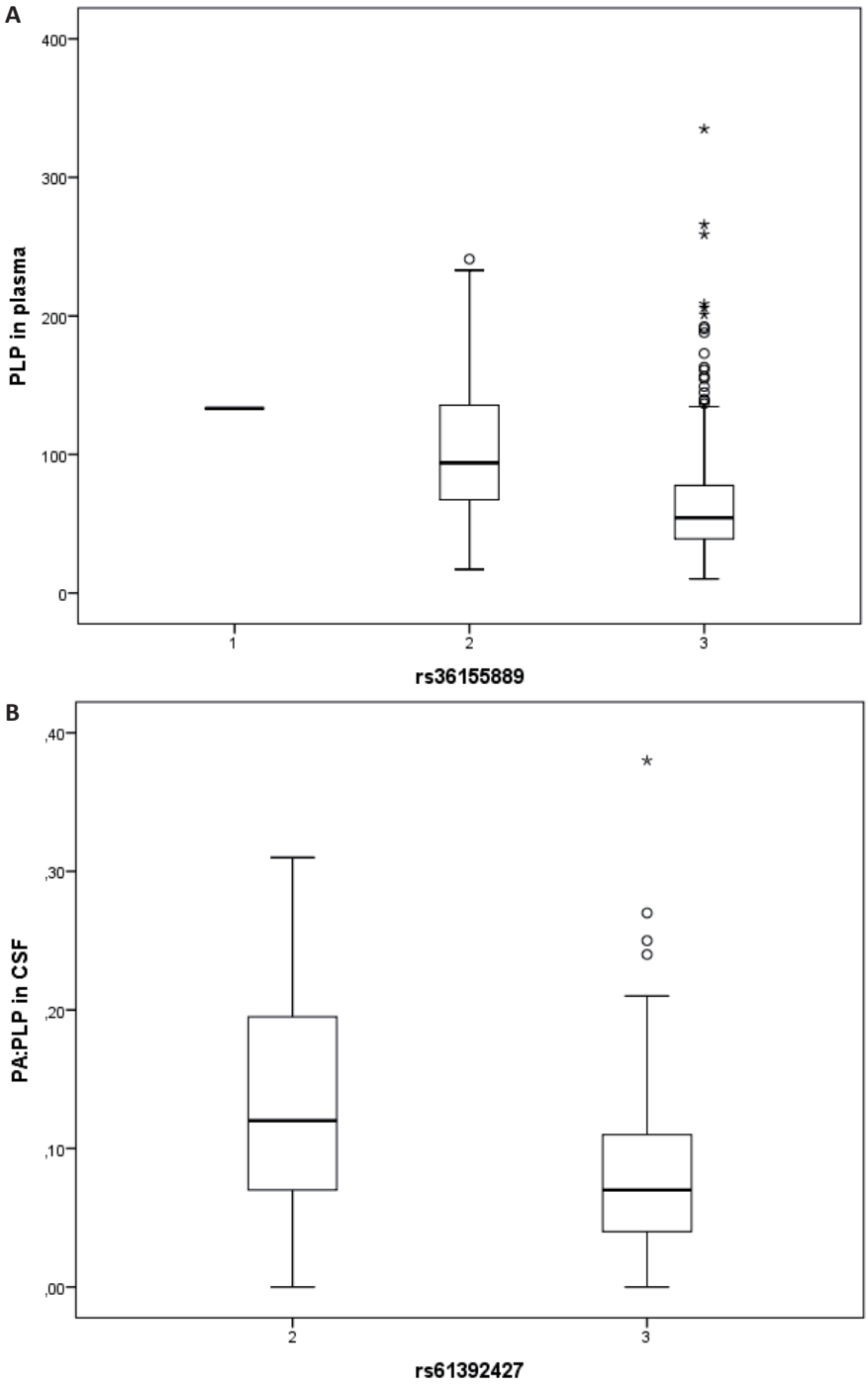

Figure 3 Relation between phenotype and genotype for rs36155889 (A) and rs61392427 (B) (1 = homozygosity for the minor allele, 2 = heterozygosity for both alleles and 3 = homozygosity for the major allele).

B6 vitamer concentrations and ratios in CSF

In CSF, genome-wide significant associations were found for ratios between PL and PM, PLP and PM, PA and PLP and for the ratio between PA and PM, as described below. No genome-wide significant associations were found for concentrations of B6 vitamers in CSF.

Ratios between PL and PM and between PLP and PM in CSF

One SNP on chromosome 9 (rs9330057; Table 3), 2.92 kb downstream of *AQP7P4* (aquaporin 7 pseudogene 4), showed a genome-wide significant association with the ratio between PL and PM in CSF (beta = -0.27 log, p = 1.56E-08; Supplementary Figure 1B) as well as with the ratio between PLP and PM in CSF (beta = -0.30 log, p = 4.22E-08; Supplementary Figure 1B). The median ratio between PL and PM in CSF of subjects heterozygous for both alleles of rs9330057 (*n*=40) was only 60% of the median ratio between PL and PM in CSF of subjects homozygous for the major allele (*n*=240, p < 5.00E-07).

Ratio between PA and PLP in CSF

For the ratio between PA and PLP in CSF, ten SNPs on chromosome 10 showed genome-wide significant associations (beta = 0.003 log, p = 1.29E-08 – 4.16E-08; Table 3). They were all located between *SNRPD2P1* (small nuclear ribonucleoprotein D2 pseudogene 1) and *HTR7* (5-hydroxytryptamine (serotonin) receptor 7, adenylate cyclase-coupled). Since these SNPs are in strong linkage disequilibrium (Figure 2B; Supplementary Figure 1C), they probably represent the same signal. The median ratio between PA and PLP in CSF of subjects heterozygous for both alleles of the most significant SNP (rs61392427, *n*=35) was approximately 70% higher than in CSF of subjects homozygous for the major allele (*n*=268, p < 2.15E-05; Figure 3B).

Ratio between PA and PM in CSF

Three SNPs in and near *C17Orf53* (chromosome 17 open reading frame 53) on chromosome 17 showed genome-wide significant associations with the ratio between PA and PM in CSF (beta = 0.09 – 0.10 log, p = 3.38E-09 – 1.56E-08; Table 3; Supplementary Figure 1D).

B6 vitamer ratios between CSF and plasma

Four SNPs on chromosome 12, all located in an intronic region of *ZDHHC17* (zinc finger, DHHC-type containing 17) were genome-wide significantly associated with the ratio of PA between CSF and plasma (beta = 0.002 log, p = 4.96E-08; Table 3; Supplementary Figure 1E).

No genome-wide significant associations were found for ratios of PL and PLP between CSF and plasma.

Suggestively significant associations

Several SNPs showed suggestively significant (p < 1.0E-06) associations with different phenotypes, as described in the Supplementary Results. See Supplementary Table 1 for a detailed overview.

SNPs in genes involved in vitamin B6 metabolism

The genes known to be involved in vitamin B6 metabolism (*ALPL*, *PDXK*, *PNPO* and *PDXP*) were studied for their association with B6 vitamer (PL, PLP, PM) and PA concentrations and ratios. In addition, we looked at SNPs in the *ALDH7A1* gene encoding antiquitin, since a deficiency of this enzyme causes functional vitamin B6 deficiency. None of the SNPs in these genes, however, showed associations at a genome-wide (p < 5.0E-08) or suggestive (p < 1.0E-06) significance level. We therefore used an informative threshold of p < 1.0E-03 (Supplementary Table 2).

As a result, we found SNPs in both *ALPL* and *PDXK*, which were associated with different phenotypes (Supplementary Table 2). The strongest association for the *ALPL* gene was found between rs4021228, in an intronic region of *ALPL*, and the concentration of PLP in CSF (beta = -0.06 log, p = 4.20E-05; Supplementary Table 2). The strongest association for the *PDXK* gene was found between rs117674831, in an intronic region of *PDXK*, and the ratio of PL between CSF and plasma (beta = 0.07 log, p = 1.68E-05; Supplementary Table 2). SNPs in *PNPO*, *PDXP* or *ALDH7A1* were not associated with either of the phenotypes (p > 1.0E-03).

DISCUSSION

Possible mechanisms determining B6 vitamer concentration differences between body fluids are metabolism and/or transport as well as yet undefined, genetic factors. In this study, we investigated B6 vitamers in plasma and CSF of a large number of healthy adults. Because of the unique sample collection and especially the large amount of CSF samples from healthy adults, we were able to compare B6 vitamer concentrations in both body fluids and investigate their association with common genetic variants.

We found a genome-wide significant association between the concentration of PLP in plasma and a SNP (rs36155889) located upstream of *SLC27A6*. Another SNP, also located near *SLC27A6*, was suggestively associated with the concentration of PLP in plasma.

The *SLC27A6* gene (OMIM *604196) encodes a long-chain fatty acid transport protein that is primarily expressed in the heart [Gimeno et al (2003)]. Relationships between fatty acids and vitamin B6 have been reported in several studies [Krajcovicová-Kudlácková et al (2004)] [Tsuge et al (2000)] [Zhao et al (October 2012)] [Zhao et al (December 2012)]. Metabolism of polyunsaturated fatty acids (PUFAs) was impaired in case of vitamin B6 deficiency [Krajcovicová-Kudlácková et al (2004)] [Zhao et al (December 2012)] due to lower activities of δ5- and δ6-desaturases as well as acyl-CoA oxidase [Krajcovicová-Kudlácková et al (2004)] [Tsuge et al (2000)]. In addition, plasma n-3 and n-6 PUFA concentrations were decreased during vitamin B6 restriction in human subjects (mean plasma PLP 21 +/- 5 nmol/L compared to 52 +/- 14 nmol/L in non-restricted subjects; *n*=23) [Zhao et al (October 2012)].

The mechanism by which vitamin B6 influences fatty acid metabolism remains to be elucidated. Likewise, there is no current explanation for the influence of *SLC27A6* on plasma PLP.

No genome-wide significant associations were found for concentrations of B6 vitamers in CSF, suggesting that these are mainly regulated by other than genetic factors. Vitamin B6 vitamers can be interconverted by specific enzymes, which are ubiquitously expressed in cells. Therefore, B6 vitamer concentrations are probably interdependent and their ratios most likely have genetic determinants. To investigate this, we tested associations of B6 vitamer ratios in plasma and CSF, and found genome-wide significance only in CSF.

It is generally known that degradation of PLP occurs by the enzymatic actions of pyridoxal phosphatase [Jang et al (2003)] (hydrolysis of PLP to PL) and PL oxidase [Merrill et al (1984)] (oxidation of PL to PA). However, our study suggests that the balance between PA and PLP in CSF is, more importantly, under regulation of another genetic factor, because

several SNPs upstream of one of the serotonin receptor genes, *HTR7*, were genome-wide significantly associated with the ratio between PA and PLP in CSF.

HTR7 (OMIM *182137) encodes a G-protein coupled serotonin receptor, which is predominantly expressed in brain and coronary artery [Bard et al (1993)]. Its prominent downstream effectors are protein kinase A and the extracellular signal-regulated kinases 1/2 [Errico et al (2001)]. A direct link between serotonin receptor activity and vitamin B6 metabolism has not been reported.

Although the enzymes involved in vitamin B6 metabolism have been elucidated at genetic and protein levels, knowledge on vitamin B6 transport is very limited. At biochemical level, there is evidence for carrier-mediated transport [Spector and Johanson (2007)], but not a single vitamin B6 transporter has been reported to date. Because the predominant B6 vitamers in plasma are PLP and PL [Van der Ham et al (2012)] [Albersen et al (2012)] [Albersen et al (submitted)] and only the unphosphorylated B6 vitamers can be transported across cell membranes, we studied the ratio of PL between CSF and plasma. We hypothesized that this ratio might be associated with SNPs in or near the gene encoding the PL transport protein in choroid plexus and/or blood brain barrier. Unfortunately, we only detected suggestively significant associations, mainly with two SNPs in an uncharacterized locus (*LOC100506272*) on chromosome 4.

To answer the question which of the enzymes known to be involved in vitamin B6 metabolism is or are dominant in determining B6 vitamer concentrations, SNPs in the genes encoding alkaline phosphatase (*ALPL*), pyridoxal kinase (*PDXK*), pyridox(am)ine phosphate oxidase (*PNPO*) and pyridoxal phosphatase (*PDXP*) were studied for their association with PL, PLP, PM and PA concentrations and ratios in and between plasma and CSF.

None of these genes showed associations at a genome-wide ($p < 5.0E-08$) or suggestive ($p < 1.0E-06$) significance level. However, several SNPs in *ALPL* were associated with the concentration of PLP in CSF and with ratios between PA and PLP as well as PLP and PL in CSF, at an informative level of $p < 1.0E-03$. For *PDXK*, several SNPs were associated with the concentration of PM in CSF and with ratios of PL and PA between CSF and plasma ($p < 1.0E-03$). Since SNPs in and near other genes did show genome-wide and/or suggestively significant associations with these phenotypes, it can be concluded that other genetic factors are more important in the regulation of B6 vitamer concentrations in plasma and CSF than the genes encoding the enzymes so far known to be involved in vitamin B6 metabolism.

In the study of Hazra et al (2009), a SNP (rs1256335) in an intronic region of *ALPL* was genome-wide significantly associated with the concentration of PLP in plasma. This SNP was not associated with any of the B6 vitamer concentrations and ratios in our study ($p >$

1.0E-03). In addition, both Hazra et al (2009) and Tanaka et al (2009) found a genome-wide significant association between rs4654748, in an intronic region of *NBPF3* (neuroblastoma breakpoint family, member 3) on chromosome 1, and plasma vitamin B6 (PLP). In our study, however, this SNP was not associated with the PLP concentration in plasma (p = 1.49E-03), nor with any of the other phenotypes, but we did find three other SNPs in a coding region of *NBPF3*, which were suggestively associated with the concentration of PLP in CSF (p = 9.36E-08 – 1.19E-07).

Compared to other GWAS performed on vitamin B6 in body fluids, which, to our knowledge, were only carried out for PLP in plasma [Hazra et al (2009)] [Tanaka et al (2009)], our sample size seems relatively small. However, our study is based on a unique collection of simultaneously drawn samples of plasma and CSF from healthy volunteers, enabling comparison between the two body fluids. Furthermore, we are the first to show GWAS data not only on PLP, but also on the other B6 vitamers (PL and PM) and the degradation product of vitamin B6 (PA).

Because four different B6 vitamers were studied for their concentrations and ratios, multiple testing is a possible drawback of our study. Our sample size however, was too small to correct for this. We therefore applied the generally accepted genome-wide significance threshold of p < 5.0E-08 [Barsh et al (2012)].

CONCLUSION

With this GWAS of B6 vitamers (PL, PLP, PM) and PA, we obtained insight in the genetic associations of B6 vitamer concentrations and ratios. The identification of several genome-wide significant SNPs at loci containing transporter and neurotransmitter receptor genes is intriguing and the mechanism by which these genes influence vitamin B6 remains to be elucidated. Besides reproducing our findings in an additional cohort of healthy human subjects, the future challenge will, of course, in general lie in increasing sample sizes and thus power as well as validity of studies investigating associations of metabolites in plasma and CSF of human subjects.

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SUPPLEMENTARY RESULTS

Several SNPs showed suggestively significant (p < 1.0E-06) associations with different phenotypes. See Supplementary Table 1 for a detailed overview.

B6 vitamer concentrations in plasma

For PLP in plasma, an additional SNP on chromosome 5 was found, also 12.3 kb upstream of *SLC27A6* (rs36177927, beta = 0.14 log, p = 3.95E-07). In addition, two other SNPs were suggestively associated with concentrations of PLP in plasma: rs17451666 on chromosome 8, 26.0 kb complementary downstream of *RPL3P9* (ribosomal protein L3 pseudogene 9) and 12.5 kb upstream of *STMN2* (stathmin-like 2) (beta = 0.12 log, p = 9.52E-07); and rs73462609 on chromosome 15, 84.0 kb downstream of *LINC00052* (long intergenic non-protein coding RNA 52) and 213 kb complementary upstream of *NTRK3* (neurotrophic tyrosine kinase, receptor, type 3) (beta = 0.11 log, p = 8.66E-07).

No suggestively significant associations were found for concentrations of the other B6 vitamers in plasma.

B6 vitamer concentrations in CSF

Two SNPs on chromosome 1, both in an intronic region of *RGS7* (regulator of G-protein signalling 7), were suggestively associated with concentrations of PL in CSF (rs1915870, beta = 0.05 log, p = 1.41E-07; rs10802946, beta = 0.05 log, p = 5.16E-07).

Three other SNPs on chromosome 1, all in a coding region of *NBPF3* (neuroblastoma breakpoint family, member 3), showed suggestively significant associations with PLP concentrations in CSF (rs3820294, rs3820293 and rs3820292; beta = 0.08 log, p = 9.36E-08 – 1.19E-07). Also suggestively associated with concentrations of PLP in CSF were three SNPs on chromosome 16, 12.6 – 12.7 kb upstream of *DYNLRB2* (dynein, light chain, roadblock-type 2) (rs12930989, rs12929931 and rs12930107; beta = 0.11 log, p = 1.58E-07).

Regarding concentrations of PM in CSF, fifteen SNPs on chromosome 2, all 120-180 kb downstream of *LOC100421125* and 265-326 kb upstream of *THSD7B* (thrombospondin, type I, domain containing 7B), showed suggestively significant associations (p = 1.92E-07 – 6.96E-07).

PA concentrations in CSF were suggestively associated with rs78824291 on chromosome 12, 34.2 kb complementary downstream of *NOS1* (nitric oxide synthase 1 (neuronal)) and 57.0 kb complementary upstream of *KSR2* (kinase suppressor of ras 2) (beta = 0.02 log, p = 8.08E-07).

Ratios between B6 vitamers in plasma

The ratio between PA and PL in plasma was suggestively associated with rs4899021 on chromosome 14, in an intronic region of *MNAT1* (menage a trois homolog 1, cyclin H assembly factor (Xenopus Laevis)) (beta = -0.12 log, p = 5.12E-07).

No suggestively significant associations were found for other ratios between B6 vitamers in plasma, except for some SNPs showing suggestively significant associations with multiple phenotypes (see below).

Ratios between B6 vitamers in CSF

Ratio between PLP and PL in CSF

Three SNPs in *LOC100996355* on chromosome 7 showed a suggestively significant association with the ratio between PLP and PL in CSF (rs2057762, rs876421 and rs2057761; beta = 0.08 log, p = 3.73E-07 – 8.10E-07). Seven other SNPs, all complementary downstream of the same *LOC100996355* and upstream of *NFE2L3* (nuclear factor (erythroid-derived 2)-like 3), were also suggestively associated with this phenotype (beta = 0.08 log, p = 8.10E-07).

Ratio between PA and PLP in CSF

In addition to the ten SNPs on chromosome 10, which were all downstream of *SNRPD2P1*, complementary upstream of *HTR7* and genome-wide significantly associated with the ratio between PA and PLP in CSF, another seven SNPs in the same region showed suggestively significant associations with this phenotype (beta = 0.002 log, p = 1.25E-07 – 2.15E-07). Furthermore, one SNP on chromosome 8, in an intronic region of *CSGALNACT1* (chondroitin sulfate N-acetylgalactosaminyltransferase 1), as well as two SNPs complementary downstream of the same *CSGALNACT1* and upstream of *INTS10* (integrator complex subunit 10), were suggestively associated with the ratio between PA and PLP in CSF (beta = 0.002 log, p = 2.23E-07 – 8.56E-07). The ratio between PA and PLP in CSF was also suggestively associated with several other SNPs on different chromosomes.

Ratio between PL and PM in CSF

The ratio between PL and PM in CSF was suggestively associated with two SNPs on chromosome 12, in an intronic region as well as 1.87 kb upstream of *ETV6* (ets variant 6), respectively (rs2724631, beta = 0.17 log, p = 7.24E-07; rs4763714, beta = 0.17 log, p = 9.21E-07). Furthermore, a suggestively significant association was found for rs12150561 on

chromosome 17, 6.35 kb downstream of *C17orf53* and 1.88 kb upstream of *ASB16* (beta = 0.22 log, p = 6.89E-07), which was already found to be genome-wide significantly associated with the ratio between PA and PM in CSF.

Ratio between PLP and PM in CSF

Both SNPs rs4736544 and rs10096294, in an intronic region of *HHLA1* on chromosome 8, showed a suggestively significant association with the ratio between PLP and PM in CSF (beta = -0.15 and -0.16 log, p = 8.17E-07 and 2.80E-07).

Ratio between PA and PM in CSF

A fourth, suggestively significant SNP was found on chromosome 17 (0.46 kb complementary downstream of *HDAC5* (histone deacetylase 5) and 17.8 kb upstream of *C17Orf53*), in the same region as three genome-wide significantly associated SNPs regarding the ratio between PA and PM in CSF (rs2129301, beta = 0.07 log, p = 2.18E-07). Also rs78852941 on chromosome 8, in an intronic region of *MFHAS1* (malignant fibrous histiocytoma amplified sequence 1), was suggestively associated with the ratio between PA and PM in CSF (beta = 0.09 log, p = 7.06E-07).

No suggestively significant associations were found for other ratios between B6 vitamers in CSF, except for some SNPs showing suggestively significant associations with multiple phenotypes (see below).

Ratios of B6 vitamers between CSF and plasma

Whereas no genome-wide significant associations were found for the ratio of PL between CSF and plasma, two SNPs in *LOC100506272* on chromosome 4 showed suggestively significant associations with this phenotype (rs7677258, beta = -0.07 log, p = 4.83E-07; rs6813185, beta = 0.07 log, p = 6.27E-07). Another SNP, rs6670440 on chromosome 1, 63.5 kb complementary downstream of *HEATR1* (HEAT repeat containing 1), 18.4 kb upstream of *ACTN2* (actinin, alpha 2) and 127 kb upstream of *MTR* (5-methyltetrahydrofolate-homocysteine methyltransferase), was also suggestively associated with the ratio of PL between CSF and plasma (beta = 0.09 log, p = 2.91E-07).

Amongst suggestively significant associations with several other SNPs on different chromosomes, the ratio of PLP between CSF and plasma was associated with rs7331922 on chromosome 13, 35.0 kb complementary downstream of *DOCK9* (DOCK9 dedicator of cytokinesis 9), 52.2 kb upstream of *RPS6P23* (ribosomal protein S6 pseudogene 23) and 69.2

kb complementary upstream of *GAPDHP22* (glyceraldehyde 3 phosphate dehydrogenase pseudogene 22) (beta = 0.13 log, p = 1.09E-07).

For the ratio of PA between CSF and plasma, another SNP in an intronic region of *ZDHHC17* on chromosome 12, in the same region as four other SNPs with a genome-wide significant association, was found to be suggestively associated (rs7309200, beta = 0.002 log, p = 3.01E-07). Furthermore and amongst others, two SNPs on chromosome 7, 56.2 kb downstream of *STAG3L4* (stromal antigen 3-like 4) and in an intronic region of *STAG3L4*, respectively, were suggestively associated with the ratio of PA between CSF and plasma (rs13438181, beta = 0.002 log, p = 3.48E-07; rs73148449, beta = 0.001 log, p = 6.59E-07).

SNPs showing suggestively significant associations with multiple phenotypes

Several SNPs showed suggestively significant associations with multiple phenotypes.

On chromosome 2, rs186118162 (37.9 kb complementary downstream of *LOC100506993*, 66.6 kb upstream of *PCGEM1* (prostate-specific transcript (non-protein coding)) and 90.8 kb upstream of *RPS17P8* (ribosomal protein S17 pseudogene 8)) showed suggestively significant associations with PA concentrations in CSF (beta = 0.04 log, p = 2.41E-07) and the ratio between PA and PL in CSF (beta = 0.001 log, p = 8.86E-07).

On chromosome 8, rs7817990 (18.6 kb complementary upstream of *ASNSP1* (asparagine synthetase pseudogene 1) was suggestively associated with PM concentrations in CSF (beta = 0.01 log, p = 1.82E-07), the ratio between PL and PM in CSF (beta = -0.28 log, p = 6.61E-08), the ratio between PLP and PM in CSF (beta = -0.32 log, p = 8.95E-08) and the ratio of PA between CSF and plasma (beta = 0.002 log, p = 8.93E-07).

On chromosome 9, rs9330057 (2.92 kb downstream of *AQP7P4* (aquaporin 7 pseudogene 4), 19.3 kb complementary downstream of *LOC286297* and 219 kb complementary upstream of *AQP7P1* (aquaporin 7 pseudogene 1)) showed suggestively significant associations with PM concentrations in CSF (beta = 0.009 log, p = 4.56E-07) and with the ratio between PA and PL in plasma (beta = -0.22 log, p = 6.70E-07). In addition, this SNP was already found to be genome-wide significantly associated with the ratio between PL and PM and the ratio between PLP and PM in CSF.

SUPPLEMENTARY TABLE

Supplementary Table 1 Suggestively significant (p < 1.0E-06) associations for B6 vitamer (PL, PLP, PM) and PA concentrations and ratios in and between plasma and CSF.

Phenotype	SNP	Chr	Position (bp)	Allele	MAF	*Locus & Function*	Number	Beta	Significance (p-value)
PL in CSF	rs1915870	1	241444781	C/T	0.45	*RGS7* (intronic)	303	0.05 log	1.41E-07
	rs10802946		241451495		0.46				5.16E-07
PLP in CSF	rs3820294	1	21806619	C/T	0.32	*NBPF3* (coding)	303	0.08 log	9.36E-08
	rs3820293		21806621	G/T					
	rs3820292		21806624	G/A	0.33				1.19E-07
	rs12930989	16	80562160	A/C	0.14	*DYNLRB2* (12.7 kb upstream)		0.11 log	1.58E-07
	rs12929931		80562191	T/G					
	rs12930107		80562285			*DYNLRB2* (12.6 kb upstream)			
	rs62049896		80607519	C/G	0.16	*DYNLRB2* (23.0 kb downstream) *CDYL2* (30.2 kb upstream (c))		0.10 log	7.12E-07
PLP in plasma	rs36177927	5	128288919	C/T	0.12	*SLC27A6* (12.3 kb upstream)	367	0.14 log	3.95E-07
	rs17451666	8	80510544		0.14	*RPL3P9* (26.0 kb downstream (c)) *STMN2* (12.5 kb upstream)		0.12 log	9.52E-07
	rs73462609	15	88206894		0.17	*LINC00052* (84.0 kb downstream) *NTRK3* (213 kb upstream (c))		0.11 log	8.66E-07

PM in CSF	rs7595457	2	137483522	G/A	0.50	*LOC100421125* (180 kb downstream) *THSD7B* (265 kb upstream)	303	-0.005 log	1.92E-07
	rs4263167		137446163		0.49	*LOC100421125* (143 kb downstream) *THSD7B* (302 kb upstream)			2.77E-07
	rs12185551		137427080	A/G		*LOC100421125* (124 kb downstream) *THSD7B* (321 kb upstream)			2.91E-07
	rs10181066		137433433	T/G	0.48	*LOC100421125* (130 kb downstream) *THSD7B* (315 kb upstream)		0.005 log	3.45E-07
	rs34027802		137425281	A/G	0.50	*LOC100421125* (122 kb downstream) *THSD7B* (323 kb upstream)			3.95E-07
	rs4375925		137468911	G/A	0.42	*LOC100421125* (166 kb downstream) *THSD7B* (280 kb upstream)		-0.005 log	4.94E-07
	rs17700151		137433150	G/T	0.49	*LOC100421125* (130 kb downstream) *THSD7B* (315 kb upstream)		0.004 log	5.26E-07
	rs10166810		137441181	T/C		*LOC100421125* (138 kb downstream) *THSD7B* (307 kb upstream)			
	rs4419275		137447448		0.36	*LOC100421125* (144 kb downstream) *THSD7B* (301 kb upstream)		-0.005 log	5.84E-07
	rs6719768		137471525	G/A	0.33	*LOC100421125* (168 kb downstream) *THSD7B* (277 kb upstream)			6.00E-07
	rs10170108		137449956		0.40	*LOC100421125* (147 kb downstream) *THSD7B* (299 kb upstream)			6.28E-07
	rs11691925		137457675	C/A		*LOC100421125* (155 kb downstream) *THSD7B* (291 kb upstream)			
	rs4528819		137473134	C/T		*LOC100421125* (170 kb downstream) *THSD7B* (275 kb upstream)			
	rs1427609		137474450			*LOC100421125* (171 kb downstream) *THSD7B* (274 kb upstream)			
	rs6715343		137422606	T/C	0.48	*LOC100421125* (120 kb downstream) *THSD7B* (326 kb upstream)		0.004 log	6.96E-07
	rs12212329	6	169271754	A/G	0.24	*SMOC2* (203 kb downstream) *THBS2* (344 kb upstream (c))	303	0.005 log	8.82E-07
	rs7817990	8	47472201	A/C	0.06	*ASNSP1* (18.6 kb upstream (c))	250	0.01 log	1.82E-07
	rs9330057	9	67051352	A/C	0.06	*AQP7P4* (2.92 kb downstream) *LOC286297* (19.3 kb downstream (c)) *AQP7P1* (219 kb upstream (c))	285	0.009 log	4.56E-07
PA in CSF	rs186118162	2	193547971	C/T	0.05	*LOC100506993* (37.9 kb downstream (c)) *PCGEM1* (66.6 kb upstream) *RPS17P8* (90.8 kb upstream)	303	0.04 log	2.41E-07
	rs78824291	12	117833785	C/T	0.35	*NOS1* (34.2 kb downstream (c)) *KSR2* (57.0 kb upstream (c))		0.02 log	8.08E-07

PL:PM in CSF	rs11735696	4	189892701	G/A	0.42	*LOC285442* (230 kb downstream) *HSP90AA4P* (502 kb upstream)	297	-0.12 log	3.87E-07
	rs7817990	8	47472201	A/C	0.06	*ASNSP1* (18.6 kb upstream (c))	246	-0.26 log	8.34E-07
	rs2724631	12	11810846	C/T	0.14	*ETV6* (intronic)	297	0.17 log	7.24E-07
	rs4763714	12	11800917	T/C	0.13	*ETV6* (1.87 kb upstream)	297	0.17 log	9.21E-07
	rs12150561	17	42246196	C/T	0.06	*C17orf53* (6.35 kb downstream) *ASB16* (1.88 kb upstream)	297	0.22 log	6.89E-07
PLP:PL in CSF	rs2057762	7	26170009	T/C	0.26	*LOC100996355* (?)	303	0.08 log	3.73E-07
	rs876421		26167109		0.27				4.05E-07
	rs2057761		26170112		0.26				8.10E-07
	rs7781648		26171175	A/G		*LOC100996355* (0.77 kb downstream (c)) *NFE2L3* (20.7 kb upstream)			
	rs10277536		26172493	C/G		*LOC100996355* (2.08 kb downstream (c)) *NFE2L3* (19.4 kb upstream)			
	rs75064424		26175373	A/G		*LOC100996355* (4.96 kb downstream (c)) *NFE2L3* (16.5 kb upstream)			
	rs2057765		26175542	A/C		*LOC100996355* (5.13 kb downstream (c)) *NFE2L3* (16.3 kb upstream)			
	rs2057764		26175652	T/C		*LOC100996355* (5.24 kb downstream (c)) *NFE2L3* (16.2 kb upstream)			
	rs4719859		26177973	A/G		*LOC100996355* (7.56 kb downstream (c)) *NFE2L3* (13.9 kb upstream)			
	rs4719860		26178183	T/C		*LOC100996355* (7.77 kb downstream (c)) *NFE2L3* (13.7 kb upstream)			
PLP:PM in CSF	rs7817990	8	47472201	A/C	0.06	*ASNSP1* (18.6 kb upstream (c))	246	-0.32 log	8.95E-08
	rs4736544		133100786	G/A	0.20	*HHLA1* (intronic)	297	-0.15 log	8.17E-07
	rs10096294		133109186	C/T			297	-0.16 log	2.80E-07
	rs191288503	19	10159931	G/A	0.06	*C3P1* (intronic)	297	0.28 log	1.78E-07
PA:PL in CSF	rs186118162	2	193547971	C/T	0.05	*LOC100506993* (37.9 kb downstream (c)) *PCGEM1* (66.6 kb upstream) *RPS17P8* (90.8 kb upstream)	303	0.001 log	8.86E-07
PA:PL in plasma	rs9330057	9	67051352	A/C	0.06	*AQP7P4* (2.92 kb downstream) *LOC286297* (19.3 kb downstream (c)) *AQP7P1* (219 kb upstream (c))	349	-0.22 log	6.70E-07
	rs4899021	14	61321953	G/T	0.19	*MNAT1* (intronic)	367	-0.12 log	5.12E-07

PA:PLP in CSF	rs12117144	1	25335222	T/C	0.15	*RUNX3* (43.7 kb downstream (c)) *MIR4425* (14.8 kb upstream)	303	0.002 log	4.28E-07
	rs11800754		25336654	G/A		*RUNX3* (45.2 kb downstream (c)) *MIR4425* (13.3 kb upstream)			
	rs1555220		25337587	A/G		*RUNX3* (46.1 kb downstream (c)) *MIR4425* (12.4 kb upstream)			
	rs1007354		25337896	T/C		*RUNX3* (46.4 kb downstream (c)) *MIR4425* (12.1 kb upstream)			
	rs1007353		25337938	C/T		*RUNX3* (46.4 kb downstream (c)) *MIR4425* (12.1 kb upstream)			
	rs11249238		25339779	A/G		*RUNX3* (48.3 kb downstream (c)) *MIR4425* (10.2 kb upstream)			
	rs1079253		25333785		0.12	*RUNX3* (42.3 downstream (c)) *MIR4425* (16.2 kb upstream)			5.95E-07
	rs12752111		4334235	T/C		*LOC644357* (98.3 kb downstream (c)) *LOC284661* (138 kb upstream)			4.53E-07
	rs17409243	8	19539135	T/C	0.06	*CSGALNACT1* (intronic)		0.002 log	2.23E-07
	rs1492643		19548967	C/A		*CSGALNACT1* (8.71 kb downstream (c)) *INTS10* (126 kb upstream)			8.65E-07
	rs11784365		19555356	T/C		*CSGALNACT1* (15.1 kb downstream (c)) *INTS10* (120 kb upstream)			
	rs34979875	10	92099033	T/C	0.07	*SNRPD2P1* (360 kb downstream) *HTR7* (402 kb upstream (c))		0.002 log	1.25E-07
	rs35997989		92094583	C/T	0.14	*SNRPD2P1* (356 kb downstream) *HTR7* (406 kb upstream (c))			2.15E-07
	rs12571183		92095665	A/T		*SNRPD2P1* (357 kb downstream) *HTR7* (405 kb upstream (c))			
	rs12570069		92095705	C/G		*SNRPD2P1* (357 kb downstream) *HTR7* (405 kb upstream (c))			
	rs12570105		92096195	G/C		*SNRPD2P1* (357 kb downstream) *HTR7* (404 kb upstream (c))			
	rs12572364		92096395	G/A		*SNRPD2P1* (358 kb downstream) *HTR7* (404 kb upstream (c))			
	rs17093815		92104583	C/T		*SNRPD2P1* (366 kb downstream) *HTR7* (396 kb upstream (c))			
	rs12150726	18	8976685	A/G	0.10	*SOGA2* (144 kb downstream) *RPS4XP19* (43.3 kb upstream) *NDUFV2* (126 kb upstream)		0.002 log	8.68E-07

PA:PM in CSF	rs2129301	17	42201478	C/T	0.11	*HDAC5* (0.46 kb downstream (c)) *C17Orf53* (17.8 kb upstream)	297	0.07 log	2.18E-07
	rs6503492		42278406	T/A	0.13	*TMUB2* (9.31 kb downstream) *ATXN7L3* (2.88 kb downstream (c)) *UBTF* (4.00 kb upstream (c))			4.77E-07
	rs78852941	8	8686763	T/C	0.06	*MFHAS1* (intronic)		0.09 log	7.06E-07
PL in CSF:Plasma	rs6670440	1	236831349	T/C	0.18	*HEATR1* (63.5 kb downstream (c)) *ACTN2* (18.4 kb upstream) *MTR* (127 kb upstream)	290	0.09 log	2.91E-07
	rs7677258	4	188486138	T/C	0.50	*LOC100506272* (?)		-0.07 log	4.83E-07
	rs6813185		188493394	A/C	0.49			0.07 log	6.27E-07
PLP in CSF:Plasma	rs1845707	3	30305766	A/C	0.45	*RBMS3* (254 kb downstream) *TGFBR2* (342 kb upstream) *GADL1* (462 kb upstream (c))	290	0.08 log	8.87E-07
	rs76846206	4	182225501	A/G	0.16	*LINC00290* (145 kb downstream (c)) *LOC132386* (218 kb upstream)		-0.11 log	9.65E-07
	rs76522245		182229502	T/C		*LINC00290* (149 kb downstream (c)) *LOC132386* (214 kb upstream)			
	rs7331922	13	99773610	C/T	0.10	*DOCK9* (35.0 kb downstream (c)) *RPS6P23* (52.2 kb upstream) *GAPDHP22* (69.2 kb upstream (c))		0.13 log	1.09E-07
PA in CSF:Plasma	rs13438181	7	66842711	T/C	0.07	*STAG3L4* (56.2 kb downstream)	290	0.002 log	3.48E-07
	rs73148449		66781108	A/G	0.10	*STAG3L4* (intronic)		0.001 log	6.59E-07
	rs7817990	8	47472201	A/C	0.06	*ASNSP1* (18.6 kb upstream (c))	243	0.002 log	8.93E-07
	rs7309200	12	77182464	C/T	0.08	*ZDHHC17* (intronic)	290	0.002 log	3.01E-07
	rs899070		77150896	A/C	0.07	*RWDD1P3* (?)			7.78E-07
	rs79897083	13	59493700	A/G	0.08	*HMGN2P39* (90.7 kb upstream)		0.001 log	6.84E-07
	rs141947454	17	85523	G/A	0.06	*RPH3AL* (intronic)		0.002 log	5.57E-07

SNP = single nucleotide polymorphism, Chr = chromosome, bp = base pair, MAF = minor allele frequency, (c) = complementary

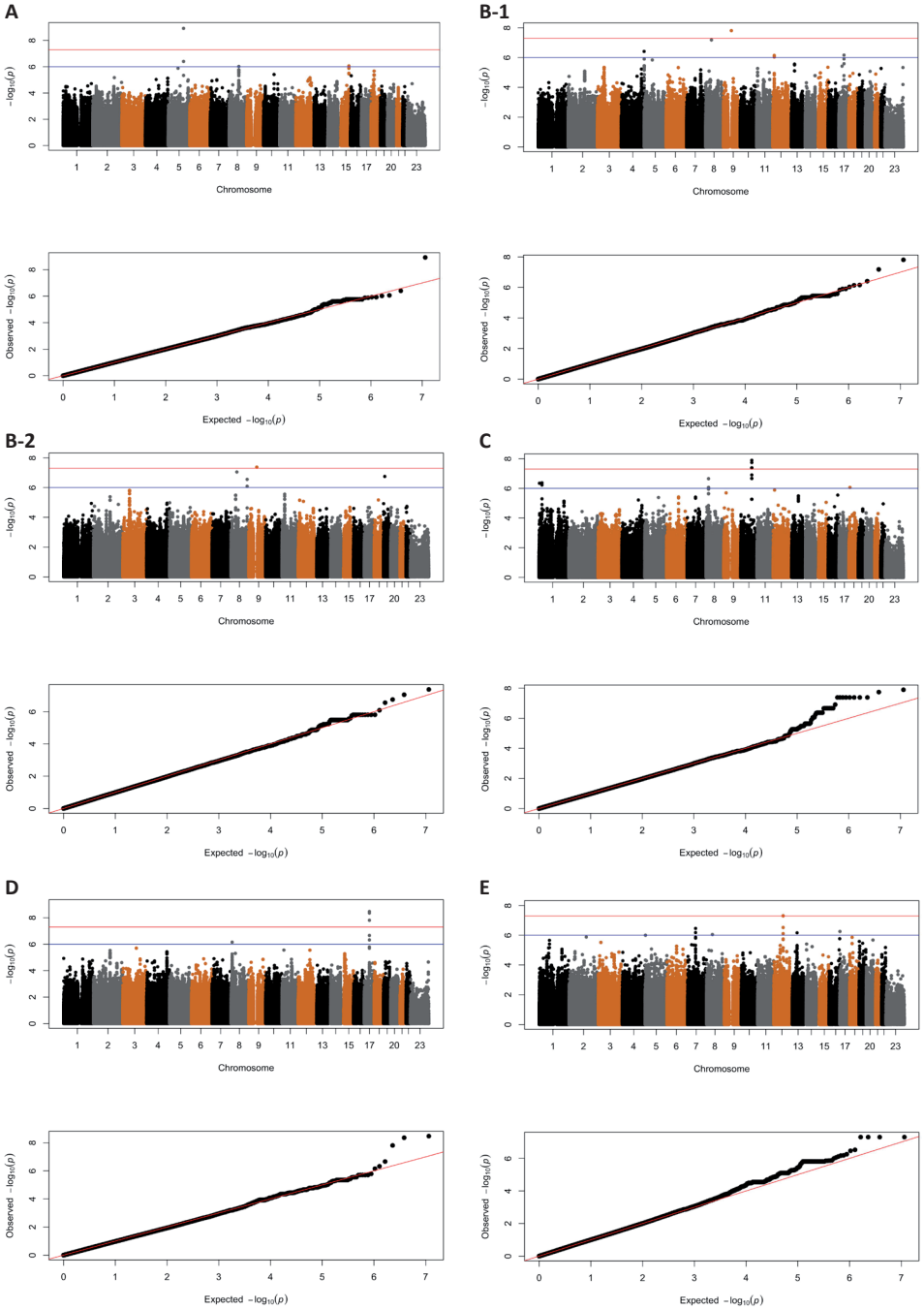
Supplementary Table 2 SNPs in the *ALPL* and *PDXK* genes known to be involved in vitamin B6 metabolism.

Phenotype	SNP	Chr	Position (bp)	*ALPL* (alkaline phosphatase)	Number	Beta	Significance (p-value)
PLP in CSF	rs4021228	1	21863691	*ALPL* (intronic)	303	-0.06 log	4.20E-05
	rs1780329		21902950			-0.07 log	2.10E-04
	rs12567402		21870213			0.06 log	3.45E-04
	rs12130255		21871800				
	rs12143671		21875097				
	rs6658127		21875390				
	rs885814		21875916				
	rs12128419		21864879				3.61E-04
	rs2275372		21900396			-0.08 log	4.01E-04
	rs2275371		21900397				
	rs3820281		21894071				4.54E-04
	rs3767151		21894104				
	rs3767150		21894214				
	rs3200254		21894735	*ALPL* (coding)			
	rs3738097		21894816	*ALPL* (intronic)			
	rs3738096		21894947				
	rs3738095		21894995				
	rs4654759		21895324				
	rs3767149		21895467				
	rs3767148		21895587				
	rs16825571		21895961				
	rs16825578		21896053				
	rs16825590		21896069				
	rs16825595		21896121				
	rs4654968		21896207				
	rs2275377		21896887				
	rs2275376		21896918				
	rs2275374		21897129				
	rs2275373		21897137				
	rs10917009		21897318				
	rs10917010		21897388				
	rs10917011		21897512				
	rs74063111		21900151				
	rs3767145		21895802				4.65E-04
	rs10917012		21898119				
	rs10917013		21898190				
	rs10917015		21898388				
	rs10917016		21898418				
	rs10917017		21899052				
	rs10917020		21899173				
	rs74063110		21900112				
	rs75829132		21900146				
	rs3200255		21900171	*ALPL* (coding)			
	rs2275369		21900444	*ALPL* (intronic)			
	rs2275368		21900583				
	rs12028278		21854997			0.05 log	6.85E-04
	rs12037984		21854925			0.05 log	6.98E-04
	rs2840331		21861656				
	rs17455136		21862253				
	rs4654969		21897893			-0.09 log	7.20E-04
	rs4654970		21897894			-0.08 log	7.47E-04
PLP:PL in CSF	rs869180		21839730	*ALPL* (intronic)		0.07 log	1.14E-04
	rs4021228		21863691			-0.06 log	1.18E-04
	rs975000		21839690			0.06 log	1.67E-04
PA:PLP in CSF	rs1772719		21904374	*ALPL* (3'utr)		0.001	5.46E-05
	rs72659192		21906604	*ALPL* (1.70 kb downstream)		log	2.13E-04

Phenotype	SNP	Chr	Position (bp)	*PDXK* (pyridoxal kinase)	Number	Beta	Significance (p-value)
PM in CSF	rs743464	21	45143064	*PDXK* (intronic)	303	0.005 log	5.24E-04
PL in CSF: Plasma	rs117674831		45161351	*PDXK* (intronic)	290	0.07 log	1.68E-05
PA in CSF: Plasma	rs73908378		45167517	*PDXK* (intronic)		0.0006 log	4.85E-04
	rs2013890		45137570	*PDXK* (1.41 kb upstream)		-0.0006 log	4.96E-04
	rs2299804		45143563	*PDXK* (intronic)			6.02E-04
	rs4819303		45143675				
	rs2838355		45144013				
	rs4819304		45144045				
	rs762402		45144492				
	rs9981480		45141115				6.46E-04
	rs80267445		45161914			0.0006 log	8.31E-04
	rs2299805		45148308			-0.0006 log	8.42E-04
	rs2838358		45151046				9.15E-04
	rs2003773		45142780				9.97E-04

SNP = single nucleotide polymorphism, Chr = chromosome, bp = base pair

SUPPLEMENTARY FIGURES



Supplementary Figure 1 Manhattan- and QQ-plots for B6 vitamer concentrations and ratios with genome-wide significant ($p < 5.0E-08$) associations. A: Concentration of PLP in plasma. B-1: Ratio between PL and PM in CSF. B-2: Ratio between PLP and PM in CSF. C: Ratio between PA and PLP in CSF. D: Ratio between PA and PM in CSF. E: Ratio of PA between CSF and plasma.

CHAPTER 6

The intestine plays a substantial role in human vitamin B6 metabolism: A Caco-2 cell model

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ABSTRACT

Vitamin B6 is present in various forms (vitamers) in the diet that need to be metabolized to pyridoxal phosphate (PLP), the active cofactor form of vitamin B6. In literature, the liver has been reported to be the major site for this conversion, whereas the exact role of the intestine remains to be elucidated. Our objective was to gain insight into the role of the intestine in human vitamin B6 metabolism.

Expression of the enzymes pyridoxal kinase (PK), pyridox(am)ine phosphate oxidase (PNPO) and PLP-phosphatase was determined in Caco-2 cells and in lysates of human intestine. Vitamin B6 uptake, conversion and excretion were studied in polarized Caco-2 cell monolayers. B6 vitamer concentrations (pyridoxine (PN), pyridoxal (PL), PLP, pyridoxamine (PM), pyridoxamine phosphate (PMP)) and pyridoxic acid (PA) were quantified by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using stable isotope-labeled internal standards.

The enzymatic system involved in vitamin B6 metabolism (PK, PNPO and PLP-phosphatase) is fully expressed in Caco-2 cells as well as in human intestine. We show uptake of PN, PM and PL by Caco-2 cells, conversion of PN and PM into PL and excretion of all three unphosphorylated B6 vitamers. In conclusion, in a Caco-2 cell model, we demonstrate that the intestine plays a substantial role in human vitamin B6 metabolism.

INTRODUCTION

Vitamin B6 is present in a wide variety of foods, like meat, fish, milk products, potatoes, beans, nuts and several fruits and vegetables [Bender (2005)]. In animal products, it is primarily found as pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP), whereas plant-derived products mostly contain pyridoxine (phosphate) (PN(P)). PN is widely used as a food supplement [Bender (2005)].

The phosphorylated B6 vitamers are hydrolysed prior to uptake, which takes place in the intestine. In the past, many studies have been published which concluded that vitamin B6 enters intestinal cells by passive diffusion [Booth and Brain (1962)] [Hamm et al (1979)] [Mehansho et al (1979)] [Middleton (1977)]. No saturation was observed and it was thought that vitamin B6 was trapped within the cell by phosphorylation and protein binding. In 2003, a carrier-mediated mechanism for PN uptake in human intestinal epithelial Caco-2 (colorectal adenocarcinoma) cells was reported [Said et al (2003)]. Uptake was inhibited by pyridoxamine (PM), but not by pyridoxal (PL) or PLP, suggesting that the transporter protein is selective for two of the three unphosphorylated B6 vitamers. In mammalian colonocytes, a PN uptake mechanism with different characteristics and inhibitable by both PM and PL(P) was found [Said et al (2008)]. Despite the biochemical characterization of vitamin B6 uptake, vitamin B6 transporter proteins and their encoding genes have not yet been elucidated.

Intracellular vitamin B6 metabolism comprises several steps. First, B6 vitamers are phosphorylated by pyridoxal kinase (PK; EC2.7.1.35). Then, PNP and PMP are oxidized to yield the active form PLP, which is catalyzed by pyridox(am)ine phosphate oxidase (PNPO; EC1.4.3.5). (Figure 1) Hydrolysis of PLP to PL by an intracellular, specific phosphatase (PLP-phosphatase; EC3.1.3.74) [Jang et al (2003)] and oxidation of PL by pyridoxal oxidase (EC1.2.3.8) [Merrill et al (1984)] constitute the degradation pathway of vitamin B6, of which the major product, pyridoxic acid (PA), is excreted in urine [Bender (2005)]. In plasma, vitamin B6 is present only as PLP and PL [Midttun et al (2009)].

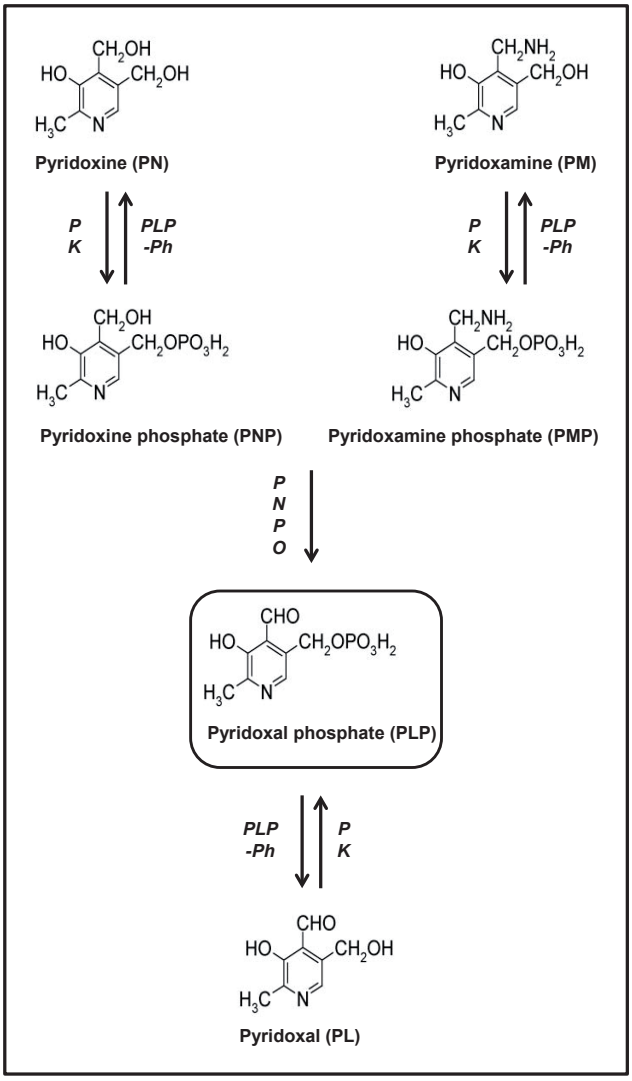

Figure 1 The different vitamin B6 vitamers and their intracellular conversions.
PK = pyridoxal kinase. PNPO = pyridox(am)ine phosphate oxidase. PLP-Ph = PLP-phosphatase.

From literature it is not clear whether dietary vitamin B6 is metabolized in the intestine or just taken up by intestinal cells and transported to the liver, where it is metabolized into PLP. The latter hypothesis is supported by many research groups through studies conducted in both humans and rodents. However, this hypothesis is not solidly founded since the intestine was bypassed through intravenous administration of vitamin B6 [Lumeng et al (1974)] or intestinal metabolism of B6 vitamers was not at all included in the described

experiments [Merrill et al (1984)] [Colombini and McCoy (1970)] [Contractor and Shane (1971)] [Johansson et al (1968)] [Johansson et al (1974)] [Lumeng et al (1980)] [Mehansho et al (1980)]. Although it is known that the liver possesses the enzymatic machinery for the formation of PLP from the other B6 vitamers and that liver cells rapidly convert all B6 vitamers into PLP, it has never been convincingly demonstrated that indeed the liver is the main location of PLP formation.

In contrast with these findings are the results of *in vivo* studies on vitamin B6 metabolism in mice [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)]. Trace amounts of [^{3}H]-PN and [^{3}H]-PM were found to be rapidly absorbed by the intestine. Shortly after administration, only [^{3}H]-PL and [^{3}H]-PLP were found in the intestine and in portal blood. This suggests that labeled PN and PM are completely converted into [^{3}H]-PL and [^{3}H]-PLP in the intestine. When ten times higher doses of [^{3}H]-PN and [^{3}H]-PM were administered, a fraction was released unchanged into the portal blood stream, suggesting that when the maximum capacity of the intestine to convert PN and PM into PL and PLP is exceeded, these B6 vitamers will be excreted by the intestine to be metabolized in the liver. [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)]

This discrepancy in literature prompted us to investigate the role of the intestine in human vitamin B6 metabolism *in vitro*. Caco-2 cells were chosen as a model system for intestinal enterocytes [Halbleib et al (2007)] because they can be grown and differentiated into polarized monolayers, creating an apical side representing the intestinal lumen and a basolateral side, corresponding with the portal blood side of the intestine. In this study, we show uptake, conversion and excretion of the unphosphorylated B6 vitamers PN, PM and PL by Caco-2 cells and confirm that all enzymes involved in vitamin B6 metabolism (PK, PNPO and PLP-phosphatase) are present in Caco-2 cells as well as in lysates of human intestine.

MATERIALS AND METHODS

Materials

Cell culture

Caco-2 and HepG2 cells were purchased from the ATCC Cell Biology Collection. Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I (20 µmol/L pyridoxine hydrochloride, 4.5 g/L D-Glucose and sodium pyruvate), B6 vitamer-free DMEM GlutaMAX-I (custom made), fetal bovine serum (FBS), penicillin-streptomycin, non-essential amino acids and trypsin-EDTA (0.5%) were purchased from Gibco (Invitrogen Life Technologies). Transwell-COL membrane

inserts (12 well cluster, 0.4 µM pore size, 1.12 cm^2 surface area, PTFE membrane) were purchased from Corning Costar Incorporated.

Western Blot

Ethylenediaminetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS; 10% *w*/*v*) and beta-mercapto-ethanol were purchased from Merck (Schuchardt, Germany). Acetone and sucrose were purchased from Sigma-Aldrich (Steinheim, Germany). Complete protease inhibitor cocktail was purchased from Roche (Woerden, The Netherlands). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Thermo Fisher Scientific Incorporated). Skim milk powder and bovine serum albumin (BSA) were purchased from Fluka Analytical (Sigma-Aldrich). Protran nitrocellulose transfer membranes were purchased from Whatman (Dassel, Germany). Enhanced chemiluminescence (ECL) Western Lightning Plus was purchased from PerkinElmer (Walthan, USA) and high performance chemiluminescence (Hyperfilm ECL) films were purchased from Amersham Biosciences (GE Healthcare).

Human normal whole tissue lysates of small intestine, colon and liver (5.0 mg/mL) in a buffer with protease inhibitor cocktail were purchased from Novus Biologicals. Mouse polyclonal antibodies against partial recombinant PK (H00008566) and PNPO (H00055163) were purchased from Abnova and a mouse monoclonal antibody against PLP-phosphatase (sc-271379) was purchased from Santa Cruz Biotechnology. A secondary goat anti-mouse HRP antibody (32430) was purchased from Pierce. A rabbit antibody against actin (A5060) was purchased from Sigma-Aldrich and a secondary goat anti-rabbit HRP antibody (31460) was purchased from Perbio Science The Netherlands BV (Thermo Fisher Scientific Incorporated).

Vitamin B6 metabolism studies and UPLC-MS/MS analysis

Pyridoxine (≥98%), pyridoxal-hydrochloride (≥99%) and pyridoxamine-dihydrochloride (≥98%) were purchased from Sigma-Aldrich. Trichloroacetic acid (TCA, >99%) was purchased from Merck KGaA (Darmstadt, Germany). The internal standards PL-hydrochloride-D_3 (99%), PN-hydrochloride-$^{13}C_4$ (99%), PA-D_2 (98%) and methyl-D_3-PLP (97%) were purchased from Buchem BV (Apeldoorn, The Netherlands). A Xevo triple quadropole mass spectrometer (TQ MS) with an electrospray ionisation (ESI) source and an Acquity UPLC were used for quantification of B6 vitamers (Waters, Manchester, UK), according to the method described by Van der Ham et al [Van der Ham et al (2012)].

Methods

Cell culture

Cells were grown and maintained in DMEM GlutaMAX-I supplemented with 10% FBS, 1% penicillin-streptomycin and 1% non-essential amino acids, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passed twice a week at >80% confluency by trypsinization with 0.05% trypsin-EDTA after washing twice with phosphate-buffered saline (PBS).

For Western Blot analysis, Caco-2 and HepG2 cells (ATCC Cell Biology Collection) were grown to confluency and Caco-2 cells were additionally differentiated for 14 days. For vitamin B6 metabolism studies, Caco-2 cells from passages 35-40 were seeded on Transwell-COL membrane inserts at a density of $1x10^5$ cells per insert. Membranes were equilibrated for one hour in DMEM GlutaMAX-I with supplements at 37°C before use. Cells were supplied with fresh DMEM GlutaMAX-I medium (with supplements) every three days. Metabolism studies of PN, PM and PL were performed at 14 days of differentiation at 37°C in a humidified atmosphere containing 5% CO_2.

Western Blot

Caco-2 and HepG2 cells were washed twice with room temperature PBS before being harvested with 1.5 mL 0.05% trypsin-EDTA. Trypsin was neutralized by addition of 8.5 mL DMEM GlutaMAX-I medium (with supplements) and supernatants were removed after centrifugation (5 min at 4000 rpm). Cell pellets were washed twice with room temperature PBS and stored at -80°C.

Cell pellets were resuspended in 250 µL of lysisbuffer (50mM Tris-HCl pH 7.5, 5mM EDTA, 150 mM NaCl, 10% (*w/v*) sucrose) containing 10 µL of Complete protease inhibitor cocktail. Samples were incubated on ice (10 min), bath-sonificated (15 min) and centrifuged at 4°C (15 min at 13000 rpm). Supernatants were used for determination of protein concentrations using the BCA protein assay kit. Proteins from Caco-2 and HepG2 cell lysates were precipitated with acetone.

Laemmli sample buffer was added to precipitated proteins of Caco-2 and HepG2 cells and to human whole tissue lysates of small intestine, colon and liver (Novus Biologicals). Equal amounts (133 µg) of protein were used. Samples were heated for 5-10 minutes at 95°C. Proteins were separated on a 10% (PK) or 15% (PNPO and PLP-phosphatase) SDS-polyacrylamide gel and subsequently blotted onto a nitrocellulose membrane. Blocking was performed in 5% skim milk.

A two-step incubation with antibody dilutions (for PK and PNPO 1:1000, for PLP-phosphatase 1:100 and for anti-mouse HRP 1:5000 (PK) or 1:1000 (PNPO and PLP-phosphatase)) in blocking buffer was performed. Actin was used as a loading control (cell lysates only), for which blots were incubated in stripping buffer (100 mM beta-mercapto-ethanol, 2% (*w/v*) SDS, 62.5 mM Tris-HCl pH 6.7) for 30 minutes at 50°C and blocked subsequently in 5% BSA. Again, a two-step incubation with antibody dilutions (for actin 1:5000 and for anti-rabbit HRP 1:10.000) was performed. Proteins were visualized on high performance chemiluminescence film using ECL Plus. Western Blotting was performed at least in duplicate for each enzyme.

Vitamin B6 metabolism studies and UPLC-MS/MS analysis

Differentiated Caco-2 cell monolayers were washed twice with PBS at room temperature and were pre-incubated for one hour in B6 vitamer-free DMEM GlutaMAX-I medium without FBS, but supplemented with 1% penicillin-streptomycin and 1% non-essential amino acids. A basal B6 vitamer profile of Caco-2 cell monolayers was determined. Uptake, metabolism and excretion of B6 vitamers was studied after addition of different (0, 100 and 1000 nmol/L) concentrations of PN, PM or PL to fresh B6 vitamer-free DMEM GlutaMAX-I medium (with supplements) in the apical compartment (0.5 mL). The basolateral medium (1.5 mL) was replaced by fresh, B6 vitamer-free medium. Apical and basolateral media were collected at 0, 6 and 48 hours. Cells were washed twice with PBS at room temperature and harvested subsequently in 1.5 mL TCA for cell lysis. Cell lysates and media were stored at -80°C until analysis. Experiments were performed in triplicate.

To study potential spontaneous changes in B6 vitamer concentrations in medium, B6 vitamer-free DMEM GlutaMAX-I with supplements and 0, 100 and 1000 nmol/L of either PN, PM or PL was placed at 37°C (humidified atmosphere; 5% CO_2) and samples were taken at 0, 6 and 48 hours. B6 vitamer concentrations were determined as described below.

Apical, basolateral and intracellular B6 vitamer concentrations (PN, PL(P), PM(P) and PA; nmol/L) were determined by a sensitive and accurate ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method using stable isotope-labeled internal standards [Van der Ham et al (2012)]. 100 µL of internal standard in TCA was added to 100 µL of medium or cell lysate. After incubation in the dark (15 min at room temperature), samples were centrifuged (5 min at 13000 rpm). 10 µL of the supernatants was used for UPLC-MS/MS analysis of the different B6 vitamers. B6 vitamer concentrations were calculated as the total amount recovered in compartment and cellular fractions (pmol).

B6 vitamer concentrations in medium were corrected for spontaneous changes due to B6 vitamer instability, of which non-enzymatic transamination of PL into PM was most prominent [Snell (1945)].

RESULTS

Expression of the enzymes involved in vitamin B6 metabolism

To investigate whether the enzymes involved in vitamin B6 metabolism (PK, PNPO and PLP-phosphatase) are expressed at protein level in Caco-2 cells and human intestinal whole tissue lysates, Western Blot analysis was performed. Human hepatocellular carcinoma (HepG2) cells and human liver whole tissue lysates were used as positive controls, as high mRNA expression of the enzymes in liver has been reported [Kang et al (2004)].

The enzymes PK, PNPO and PLP-phosphatase were present in Caco-2 cells, human whole tissue lysates of small intestine and colon, as well as in HepG2 cells and human liver whole tissue lysates. (Figure 2) All protein bands were visible at the expected heights (for PK: 35.1 kDa, for PNPO: 29.9 kDa and for PLP-phosphatase: 31.7 kDa). In Caco-2 cells, all enzymes were present from one week of differentiation on (data not shown).

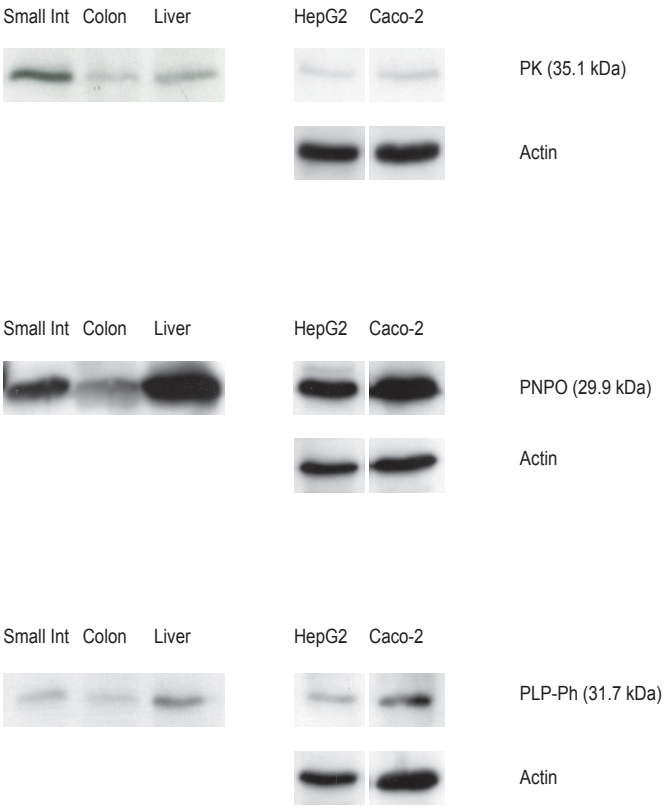

Figure 2 Expression of the enzymes involved in vitamin B6 metabolism in human cell lines and tissue lysates. Actin was used as a loading control (cell lysates only). Depicted are representations of at least duplicates.
PK = pyridoxal kinase. PNPO = pyridox(am)ine phosphate oxidase. PLP-Ph = PLP-phosphatase.
Small Int = small intestine. HepG2 = HepG2 cells. Caco-2 = Caco-2 cells.

Vitamin B6 metabolism in Caco-2 cells

To study uptake of the unphosphorylated B6 vitamers, a basal B6 vitamer profile of differentiated Caco-2 cells was determined after which the monolayers were incubated with 100 and 1000 nmol/L of PN, PM or PL. The metabolic fate of the incubated PN, PM and PL was determined by quantification of all B6 vitamers in apical and basolateral media as well as in cell lysates.

Basal B6 vitamer profile of Caco-2 cells

After growth and differentiation in medium containing 20 µmol/L PN, the B6 vitamer profile of Caco-2 cell monolayers was determined. PLP was present in the highest amount (31 pmol), next to PN (24 pmol), PL (19 pmol) and a low amount of PMP (2.3 pmol). Pre-incubation in B6 vitamer-free medium resulted in a release of PN and PL from the Caco-2 cells, but did not alter intracellular amounts of the phosphorylated B6 vitamers PLP and PMP (data not shown). PA was not present intracellularly.

Incubation with pyridoxine (PN)

During incubation of the apical side of the Caco-2 cell monolayer with PN, we observed a decrease of apical PN amounts in time, pointing to uptake of PN by Caco-2 cells. In addition, there was a slight increase in basolateral PN amounts during incubation with 1000 nmol/L of PN. (Table 1)

Table 1 Apical and basolateral amounts of PN, PM and PL in time during incubation with 100 and 1000 nmol/L of either B6 vitamer.
B6 vitamer concentrations were corrected for compartment volume yielding amounts (pmol). Depicted are means (SE) of triplicates.

Amount of B6 vitamer present (pmol)	**PN (100 nmol/L added)**	**PN (1000 nmol/L added)**	**PM (100 nmol/L added)**	**PM (1000 nmol/L added)**	**PL (100 nmol/L added)**	**PL (1000 nmol/L added)**
Apical						
t=0 hours	50.0	500	50.0	500	50.0	500
t=6 hours	38.3 (2.4)	429 (23.4)	35.6 (3.5)	397 (25.1)	30.9 (1.8)	320 (8.1)
t=48 hours	14.3 (1.0)	131 (17.2)	13.4 (0.2)	123 (3.9)	31.2 (0.6)	303 (0.9)
Basolateral						
t=0 hours	0.0	0.0	0.0	0.0	0.0	0.0
t=6 hours	1.5 (0.8)	16.2 (4.2)	0.0 (0.0)	9.4 (0.7)	15.4 (2.1)	168 (12.0)
t=48 hours	0.0 (0.0)	32.7 (2.3)	1.1 (0.2)	31.5 (2.2)	5.4 (2.3)	152 (8.8)

Metabolic conversions of intracellular PN were studied by looking at changes in amounts of the other B6 vitamers in time. Intracellularly, mainly PLP was present which remained stable during incubation with 100 nmol/L of PN but increased with approximately 23%, from 31 ± 2.9 pmol to 38 ± 1.2 pmol (in 48 hours) during incubation with 1000 nmol/L of PN. (Figure

3) In comparison, during incubation in medium devoid of B6 vitamers, intracellular amounts of PLP decreased with approximately 18%, from 31 ± 2.9 pmol to 25 ± 1.7 pmol. (Figure 3) Intracellular amounts of PMP remained low (2.0 ± 0.3, 2.2 ± 0.3 and 2.7 ± 0.3 pmol at 0, 6 and 48 hours, respectively).

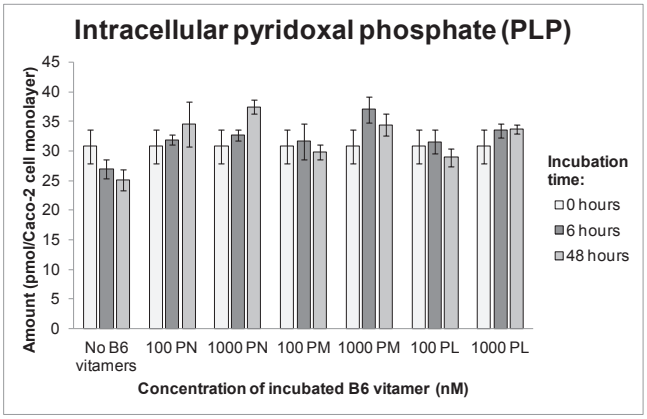

Figure 3 Intracellular amounts of PLP during incubation with no B6 vitamers, PN, PM and PL. Depicted are means ± SE of triplicates.

In medium, amounts of PL increased in time and with the concentration of incubated PN, demonstrating conversion of PN into PL by Caco-2 cells. Excretion of PL was much higher basolaterally than apically after 48 hours of incubation with PN. (Figure 4A) Amounts of PM did not change in medium during incubation with PN (data not shown). PMP and PLP were absent both apically and basolaterally.

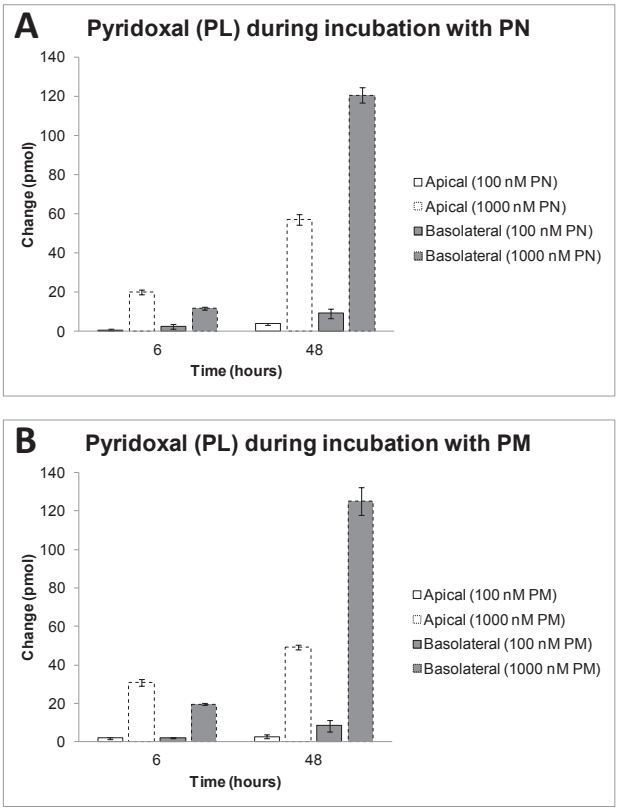

Figure 4 Changes in apical and basolateral amounts of PL during incubation with PN (A) or PM (B). Depicted are means ± SE of triplicates.

Incubation with pyridoxamine (PM)

Results of the experiments in which we incubated with PM were remarkably similar to those in which we incubated with PN. Amounts of PM decreased at the apical side of the Caco-2 cell monolayer in time and increased to some extent at the basolateral side during incubation with high PM concentrations. (Table 1)

Intracellular PLP amounts increased only very slightly during incubation with high PM concentrations. (Figure 3) Intracellular amounts of PMP remained low.

Conversion of PM into PL by Caco-2 cells was obvious from the concentration-dependent excretion of PL in medium, which was higher basolaterally than apically. (Figure 4B) Amounts of PN did not change in medium during incubation with PM (data not shown). PMP and PLP were absent both apically and basolaterally.

Incubation with pyridoxal (PL)

Results of incubation of the apical side of the Caco-2 cell monolayer with PL were different from those experiments in which we incubated with PN and PM. The decrease of apical PL amounts in time was only approximately 50% of the decrease found during incubation with PN and PM, suggesting that uptake of PL was less efficient. (Table 1) Furthermore, PL excretion at the basolateral side was approximately equal to PL uptake from the apical side of the Caco-2 cell monolayer. (Table 1)

Like in the experiments with PN and PM, mainly PLP was present intracellularly during incubation with PL and although amounts remained quite stable, they were approximately 16% higher during incubation with 1000 nmol/L compared to 100 nmol/L of PL. (Figure 3) Intracellular amounts of PMP remained low.

Furthermore, no changes in apical and basolateral amounts of PN and PM occurred during incubation with PL (data not shown), suggesting that PL is not converted into the other unphosphorylated B6 vitamers by Caco-2 cells. This is in line with the observed basolateral excretion of PL. PMP and PLP were absent both apically and basolaterally.

Formation of pyridoxic acid (PA)

Degradation of the B6 vitamers was studied by quantification of PA in apical and basolateral media as well as intracellularly. Small amounts of PA were present only in the apical medium after 48 hours of incubation with 1000 nmol/L of PN (0.9 ± 0.2 pmol), PM (1.2 ± 0.2 pmol) and PL (3.9 ± 0.1 pmol). To confirm this observation, we studied apical PA excretion during incubation with 10.000 nmol/L of PL, which resulted in at least three times higher apical than basolateral amounts of PA (data not shown). Thus, B6 vitamers can be degraded within Caco-2 cells and subsequently excreted mainly at the apical side of the Caco-2 cell monolayer during incubation with high B6 vitamer concentrations, and especially PL.

DISCUSSION

Vitamin B6 is the term used to indicate a group of unphosphorylated and phosphorylated pyridine compounds that can be enzymatically interconverted. This interconversion is important, since plant-derived foods mostly contain PN(P) whereas the biologically active cofactor is PLP. The organs that are important in this interconversion have not been irrefutably identified.

Here we report studies in a model system of human intestine, using polarized Caco-2-cell monolayers. In this system, we clearly show uptake of PN, PM and PL from the apical incubation medium. PL is not converted into PN or PM, but excreted into the basolateral compartment. In contrast, PN and PM are both converted into PL, probably by the sequential actions of PK (to give PNP and PMP), PNPO (to give PLP) and PLP-phosphatase (to give PL). The formed PL is excreted into the medium, mainly at the basolateral side (basolateral : apical excretion = approximately 2.5 : 1), suggesting a PL-specific export system in the basolateral Caco-2 cell membrane. (Figure 5)

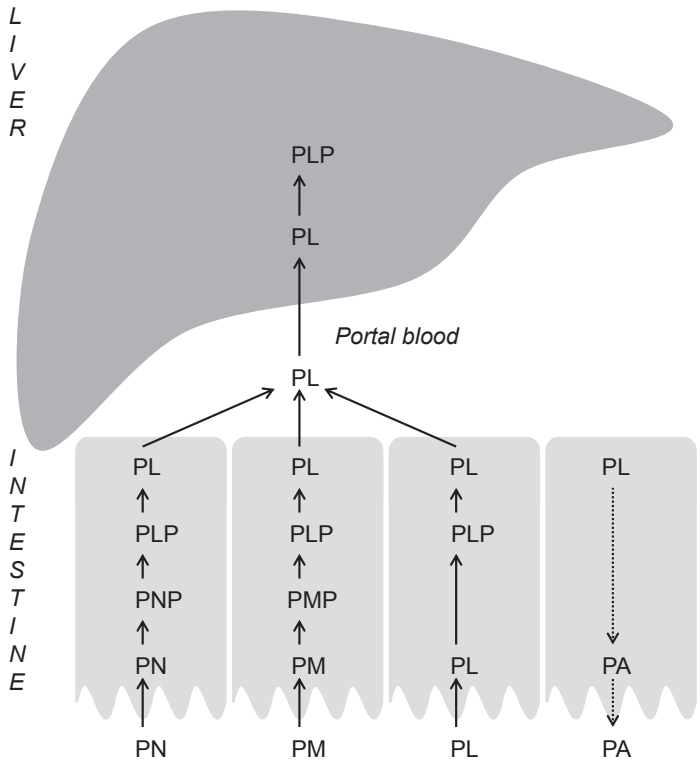

Figure 5 Hypothesis of human intestinal vitamin B6 metabolism.

Our results are in support of the findings of Sakurai et al, who showed uptake of physiological amounts of labeled PN and PM and complete conversion into PL and PLP by the mouse intestine *in vivo* [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)].

Interestingly, we also observed apical excretion of PA. Previous studies have shown expression of efflux pumps with broad substrate specificity in the apical membrane of polarized Caco-2 cells [Taipalensuu et al (2001)]. Possibly, one of these pumps may be involved in the limitation of transport of PA, the metabolic end product of PLP degradation, from intestinal cells into portal blood. (Figure 5)

In our experiments, PLP was only present intracellularly and amounts changed just to a minor extent during incubation with different concentrations of the unphosphorylated B6 vitamers. Apparently, intracellular levels of PLP are very tightly regulated and PLP is not excreted by Caco-2 cells. This seems in contrast with the studies of Sakurai et al in mice, who detected, next to PL, also PLP in portal blood after administration of PN or PM, suggesting release of both PL and PLP by the intestine [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)]. But since PLP was mainly present in the erythrocyte fraction of portal blood, it was concluded that the intestine is able to release only PL, which is converted into PLP in blood. Activity of PK in erythrocytes has been described in literature [Chern and Beutler (1975)]. In our model system of intestinal enterocytes, the basolateral compartment was used as a reflection of the portal blood side of the intestine, however, without any erythrocytes present. This might explain the absence of PLP in the basolateral medium. (Figure 5)

The enzymes involved in vitamin B6 metabolism, PK, PNPO and PLP-phosphatase, have been reported to be expressed at mRNA level in the liver and to a lesser extent also in the intestine [Kang et al (2004)]. Expression of PK, PNPO and PLP-phosphatase had not been thoroughly studied, however, at protein level. We show that the enzymatic system involved in vitamin B6 metabolism (PK, PNPO and PLP-phosphatase) is fully present both in Caco-2 cells as well as in human intestinal tissue.

In our Caco-2 cell model, basolateral PN and PM excretion only occurred in case of high apical concentrations of these B6 vitamers. Likewise, in the *in vivo* studies of Sakurai et al, only high doses of PN and PM resulted in the appearance of these B6 vitamers in portal blood [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)]. These results suggest that when the maximum capacity of the intestine to convert PN and PM into PL is exceeded, PN and PM will enter the portal circulation and will be metabolized in the liver as well.

Thus, under normal dietary circumstances, it is very likely that not PN, but mainly PL reaches the portal circulation. All other organs, including liver and brain, obtain PL from

blood and only need the enzyme PK to form PLP. This suggests that in most tissues, including brain, PNPO is not needed for PLP formation, but that it acts as a recycling enzyme in the salvage pathway of PLP rather than as a PLP synthesis enzyme.

In contrast, when high amounts of PN are administered, the capacity of the intestine is insufficient to fully metabolize all PN. Then, PN may reach the circulation and other tissues. Indeed, in plasma of subjects receiving PN supplementation, we and others detected PN in quantifiable amounts whereas it is normally undetectable in plasma (unpublished observations and [Footitt et al (2013)]). It is likely that not all of this PN is metabolized by the liver, because also in cerebrospinal fluid, high concentrations of PN were observed in PN supplemented patients [Van der Ham et al (2012)]. The consequences of this unphysiological presence of PN in plasma and cerebrospinal fluid are not yet known.

CONCLUSION

Our results shed new light on human vitamin B6 metabolism, as we demonstrate a substantial role for the intestine herein.

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CHAPTER 7

Vitamin B6 metabolism in the brain

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In preparation

ABSTRACT

Pyridoxal phosphate (PLP), the active form of vitamin B6, is crucial for normal brain development and functioning. In pyridox(am)ine phosphate oxidase (PNPO) deficiency, functional deficiency of vitamin B6 leads to severe neonatal convulsions. Supplementation of PLP can be quite successful, but developmental delay can still occur despite early treatment. Whereas the normal B6 vitamer composition of cerebrospinal fluid (CSF) is known and decreased concentrations of pyridoxal (PL) and PLP have been reported in CSF of PNPO deficient patients, the normal B6 vitamer distribution within brain cells as well as the intracellular consequences of PNPO deficiency and vitamin B6 supplementation are not known.

Uptake and metabolism of vitamin B6 by Neuro2A cells, and the dependence of the intracellular B6 vitamer distribution on the type of B6 vitamer present extracellularly, were investigated. In addition, a model of PNPO deficiency was generated by RNA interference of the *PNPO* gene and its consequences on the Neuro2A cell B6 vitamer distribution were studied. Furthermore, the *in vivo* effects of vitamin B6 supplementation were investigated by analyzing the B6 vitamer composition of CSF.

Neuro2A cells transported pyridoxine (PN), pyridoxamine (PM) and PL, metabolized these B6 vitamers and excreted PL and PLP into the medium. The intracellular B6 vitamer distribution was different for each B6 vitamer present in the medium. During exposure to PL, degradation of PL into pyridoxic acid (PA) was observed and, interestingly, Neuro2A cells excreted PN into the medium. Incubation of PNPO depleted Neuro2A cells with PN resulted in accumulation of PN and pyridoxine phosphate, whereas formation of PL and PLP was lower than in control cells. In CSF of children supplemented with PN or PLP, strongly elevated concentrations of PN, PM, PL and PA were detected, whereas concentrations of PLP were at the upper limit of normal.

Neuro2A cells take up, metabolize and excrete vitamin B6. PNPO deficiency *in vitro* not only results in lowered PLP, but also in a disbalance between B6 vitamers. The effects of high dose vitamin B6 supplementation are evident in CSF, in which extreme elevations of several B6 vitamers were found. Our findings shed light on both physiology and pathophysiology of vitamin B6 metabolism in the brain and are promising with respect to optimizing treatment regimes in case of functional vitamin B6 deficiency.

INTRODUCTION

Pyridoxal phosphate (PLP), the catalytically active form of vitamin B6, is involved in the metabolism of mostly amino acids and neurotransmitters. Consequently, PLP plays a crucial role in normal brain development and functioning. Formation of PLP from the other B6 vitamers takes place through the actions of pyridoxal kinase (PDXK) and pyridox(am)ine phosphate oxidase (PNPO) (Figure 1).

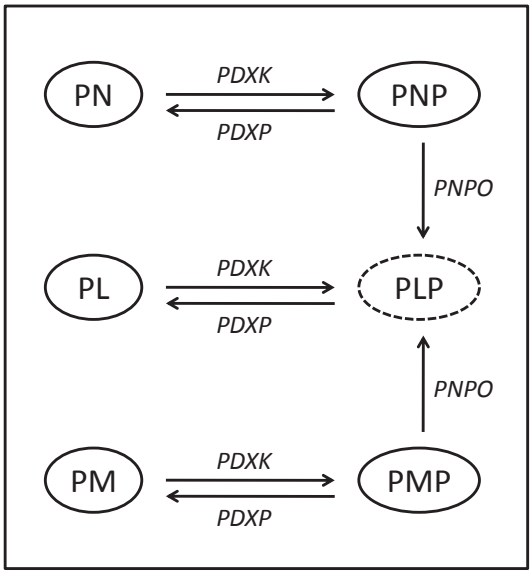

Figure 1 The different vitamin B6 vitamers and their intracellular conversions.
PN = pyridoxine. PNP = pyridoxine phosphate. PL = pyridoxal. PLP = pyridoxal phosphate. PM = pyridoxamine. PMP = pyridoxamine phosphate. *PDXK* = pyridoxal kinase. *PNPO* = pyridox(am)ine phosphate oxidase. *PDXP* = vitamin B6-specific phosphatase.
The degradation product of vitamin B6, pyridoxic acid (PA), is not depicted.

In specific inherited metabolic disorders, a functional deficiency of vitamin B6 occurs. A deficiency of PNPO (OMIM #610090) [Mills et al (2005)] leads to severe neonatal convulsions, which can be terminated by PLP administration. Pyridoxine dependent epilepsy (α-AASA dehydrogenase (antiquitin) deficiency; OMIM #266100) [Mills et al (2006)] has a very different etiology, as PLP is inactivated by an accumulating intermediate of hampered lysine degradation. This disorder can be treated with PLP as well as pyridoxine (PN).

The effects of PNPO and antiquitin deficiencies on intracellular vitamin B6 metabolism are not known, nor are the optimal treatment strategies for both disorders. In fact, the use of PN or PLP is quite successful in some cases, but only partly effective in others. A substantial number of affected children has developmental delay and neurological symptoms, despite early treatment with high doses [Bok et al (2012)] [Stockler et al (2011)]. The reason for this lack of complete success is not known. In healthy individuals, ingestion of large doses of PN is toxic and leads to polyneuropathy [Jortner (2000)]. The mechanism of this neurotoxicity has not yet been elucidated.

From our previous studies [Albersen et al (2012)] [Albersen et al (submitted)] [Van der Ham et al (2012)], it is known that the dominant B6 vitamers in human plasma and cerebrospinal fluid (CSF) are pyridoxal (PL) and PLP. PN is not detectable in plasma and CSF, unless subjects are supplemented with PN [Van der Ham et al (2012)]. Transport of PL across the membranes of brain cells and choroid plexus can either occur directly or is preceded by hydrolysis of PLP through the membrane-bound enzyme alkaline phosphatase (ALPL) [Spector and Greenwald (1978)] [Waymire et al (1995)]. Despite the fact that there is evidence for carrier-mediated transport of vitamin B6 at the biochemical level [Spector and Johanson (2007)], a vitamin B6 transport protein has not yet been characterized.

Although B6 vitamer concentrations in plasma and CSF have been published, the normal B6 vitamer distribution within brain cells is not known. In addition, there is no literature on the intracellular effects of functional vitamin B6 deficiency and vitamin B6 supplementation, while PN-related neurotoxicity has been reported. In order to optimize diagnosis, treatment and possibly also outcome of PNPO deficiency, we investigated whether vitamin B6 is taken up and metabolized by neuronal cells. We used Neuro2A (mouse neuroblastoma) cells, because this cell line has been reported to be a good model system for the study of neuronal growth and development [Thiele (1998)] [Yanaka et al (2007)]. We studied the extent to which the intracellular B6 vitamer distribution is dependent on the type of B6 vitamer present extracellularly and compared our findings with observations in CSF of children supplemented with vitamin B6. In addition, we studied the consequences of a deficiency of PNPO (by RNA interference of the *PNPO* gene) and incubation with PN on the B6 vitamer distribution within Neuro2A cells.

MATERIALS AND METHODS

Materials

Cell culture

Neuro2A cells were purchased from the ATCC Cell Biology Collection. Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I (20 µmol/L pyridoxine hydrochloride, 4.5 g/L D-Glucose and sodium pyruvate), B6 vitamer-free DMEM GlutaMAX-I (custom made), fetal bovine serum (FBS), penicillin-streptomycin (P-S) and trypsin-EDTA (0.5%) were purchased from Gibco (Life Technologies).

Western Blot of PDXK, PNPO and PDXP

Ethylenediaminetetra-acetic acid (EDTA) and sodium dodecyl sulphate (SDS; 10% *w*/*v*) were purchased from Merck (Schuchardt, Germany). Sucrose was purchased from Sigma-Aldrich (Steinheim, Germany). Complete protease inhibitor cocktail was purchased from Roche (Woerden, The Netherlands). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Thermo Fisher Scientific Incorporated). Skim milk powder was purchased from Fluka Analytical (Sigma-Aldrich). Protran nitrocellulose transfer membranes were purchased from Whatman (Dassel, Germany). Enhanced chemiluminescence (ECL) Western Lightning Plus was purchased from PerkinElmer (Walthan, USA) and high performance chemiluminescence (Hyperfilm ECL) films were purchased from Amersham Biosciences (GE Healthcare).

A mouse polyclonal antibody against partial recombinant PDXK (H00008566) was purchased from Abnova. A goat polyclonal antibody against PNPO (sc-82319) and a mouse monoclonal antibody against PDXP (sc-271379) were purchased from Santa Cruz Biotechnology. A secondary goat anti-mouse HRP antibody (32430) was purchased from Pierce and a secondary rabbit anti-goat HRP antibody (P0449) was purchased from Dako. A rabbit antibody against Actin (A5060) was purchased from Sigma-Aldrich and a secondary goat anti-rabbit HRP antibody (31460) was purchased from Perbio Science The Netherlands BV (Thermo Fisher Scientific Incorporated).

Vitamin B6 uptake and metabolism studies and UPLC-MS/MS analysis of B6 vitamers

Pyridoxine (≥98%), pyridoxal-hydrochloride (≥99%) and pyridoxamine-dihydrochloride (≥98%) were purchased from Sigma-Aldrich. Trichloroacetic acid (TCA, >99%) was purchased from Merck KGaA (Darmstadt, Germany). The internal standards PL-hydrochloride-D_3 (99%),

PN-hydrochloride-$^{13}C_4$ (99%), PA-D_2 (98%) and methyl-D_3-PLP (97%) were purchased from Buchem BV (Apeldoorn, The Netherlands). A Xevo triple quadropole mass spectrometer (TQ MS) with an electrospray ionisation (ESI) source and an Acquity UPLC were used for quantification of B6 vitamers (Waters, Manchester, UK), according to the method of Van der Ham et al [Van der Ham et al (2012)].

RNA interference of the PNPO gene

A set of 4 small interfering (si) RNAs targeting the mouse *PNPO* gene (ON-TARGETplus LQ-052058-01; no's 9-12), a non-targeting siRNA pool (ON-TARGETplus D001810-10) and a 5x siRNA buffer were purchased from Dharmacon (Thermo Fisher Scientific Incorporated). Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Life Technologies). Opti-MEM I + GlutaMAX-I (reduced serum medium) was purchased from Gibco (Life Technologies).

Methods

Cell culture

Neuro2A cells were grown and maintained in DMEM GlutaMAX-I supplemented with 10% FBS and 1% P-S, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passed at ≥70% confluency by trypsinization.

Western Blot of PDXK, PNPO and PDXP

For Western Blot analysis, Neuro2A cells were grown to ≥70% confluency. Cells were washed twice with room temperature (RT) phosphate-buffered saline (PBS) before being harvested.

Cell pellets were resuspended in 250µL of lysisbuffer (50mM Tris-HCl pH 7.5, 5mM EDTA, 150mM NaCl, 10% (*w/v*) sucrose) containing 10µL of Complete protease inhibitor cocktail. Samples were incubated on ice (10 min), bath-sonificated (15 min) and centrifuged at 4°C (15 min at 13000 rpm). Supernatants were used for determination of protein concentrations using the BCA protein assay kit and subsequently for Western Blot analysis.

Laemmli sample buffer was added to 135µg of protein from Neuro2A cell lysates. Proteins were denatured for 5-10 minutes at 95°C, separated on a 15% SDS-polyacrylamide gel and subsequently blotted onto a nitrocellulose membrane. Blocking (60 min at RT) was performed in 5% skim milk.

Antibody dilutions were as follows: anti-PDXK 1:1000, anti-PNPO 1:1000, anti-PDXP 1:100, anti-mouse 1:1000, anti-goat 1:1000, anti-Actin 1:2500 and anti-rabbit 1:5000.

Proteins were visualized on high performance chemiluminescence film using ECL Plus. Western Blotting was performed in at least duplicate for each enzyme.

Vitamin B6 uptake and metabolism studies

Confluent (≥70%) Neuro2A cells were washed twice with RT PBS and were pre-incubated for 60 min at 37°C with B6 vitamer-free DMEM GlutaMAX-I (+1% P-S). Cells were passed and grown in B6 vitamer-free DMEM GlutaMAX-I (+5% FBS and 1% P-S) to which either no vitamin B6 or 20 µmol/L of PN, pyridoxamine (PM) or PL was added (passage 16; 6-well format). Every 24 hours, media were sampled and replaced by freshly prepared media using stock solutions of PN, PM and PL, which were stored protected from light and at 4°C.

Seventy-six hours after passing, Neuro2A cells were ≥70% confluent. After sampling of media, cells were washed twice with cold PBS and lysates were obtained by scraping in TCA. For determination of protein concentrations, an additional triplicate of cells for each condition was harvested.

To study potential spontaneous changes in B6 vitamer concentrations in medium, B6 vitamer-free DMEM GlutaMAX-I (+5% FBS and 1% P-S) without vitamin B6 and with 20 µmol/L of either PN, PM or PL was placed at 37°C for 76 hours as well and samples were taken every 24 hours.

B6 vitamer concentrations were corrected for volume and expressed as the total amount present in medium (pmol) and intracellularly (pmol/mg of protein). In addition, B6 vitamers in medium were corrected for amounts detected in fresh media as well as for spontaneous changes due to B6 vitamer instability and are presented cumulatively for each time point.

RNA interference of the PNPO gene

Neuro2A cells (passage 23) were grown to 40% confluency. Medium was refreshed at the start of siRNA transfection (DMEM GlutaMAX-I +10% FBS and 1% P-S). siRNA no's 9-12 (hereafter referred to as siRNA no's 1-4) targeting the mouse *PNPO* gene and the non-targeting siRNA pool (hereafter named 'siRNA control') were mixed with the Lipofectamine RNAiMAX transfection reagent in Opti-MEM I and added to the cells (siRNA end concentration 50 nmol/L; 6-well format, duplicates for each condition).

Forty-eight hours after siRNA transfection, Neuro2A cells were washed twice with RT PBS and were pre-incubated for 60 min at 37°C with B6 vitamer-free DMEM GlutaMAX-I (+5% FBS and 1% P-S). Subsequently and for each condition, a duplicate of cells was washed twice with cold PBS and lysates were obtained by scraping in TCA (t=0 hours). For determination

of protein concentrations and for Western Blot analysis, an additional duplicate of cells for each condition was harvested.

All other duplicates for each condition were washed twice with RT PBS and were incubated with B6 vitamer-free DMEM GlutaMAX-I (+5% FBS and 1% P-S) to which 20 µmol/L of PN was added. After four hours, media were sampled and after washing twice with cold PBS, cell lysates were obtained (t=4 hours).

To study potential spontaneous changes in B6 vitamer concentrations in medium, B6 vitamer-free DMEM GlutaMAX-I (+5% FBS and 1% P-S) with 20 µmol/L of PN was placed for four hours at 37°C as well.

B6 vitamer concentrations were corrected for volume and expressed as the total amount present in medium (pmol) and intracellularly (pmol/mg of protein). In addition, B6 vitamers in medium were corrected for amounts detected in fresh media and for spontaneous changes due to B6 vitamer instability.

To study the expression (and knockdown) of PNPO in Neuro2A cells after RNA interference of the *PNPO* gene, Western Blot analysis was performed using 13µg of protein for each condition. The expression of PNPO in cells transfected with siRNAs 1-4 was normalized to the expression of PNPO in cells transfected with control siRNA and to Actin expression (ImageQuant TL 7.0 software, GE Healthcare Life Sciences).

CSF of human subjects with vitamin B6 supplementation

To study the effect of treatment with vitamin B6 on B6 vitamer concentrations in CSF of human subjects, remnant CSF samples of children supplemented with PN (*n*=3) or PLP (*n*=2) were collected. Ratios were calculated between concentrations of PLP and PL as well as between concentrations of PA and PL.

UPLC-MS/MS analysis of B6 vitamers

CSF samples, cell lysates and media were stored at -80°C until analysis. Experiments were performed in triplicate, unless specified otherwise.

B6 vitamer (PN, pyridoxine phosphate (PNP), PL, PLP, PM, pyridoxamine phosphate (PMP) and pyridoxic acid (PA)) concentrations in CSF were determined by UPLC-MS/MS using stable isotope-labeled internal standards [Van der Ham et al (2012)]. For quantification of intracellular and medium B6 vitamers, the method of Van der Ham et al was adapted: 100µL of internal standard in TCA was added to 100µL of medium or cell lysate. After incubation in the dark (15 min at RT), samples were centrifuged (5 min at 13000 rpm) and 10µL of the supernatants was used for B6 vitamer quantification.

RESULTS

Uptake, metabolism and excretion of B6 vitamers by Neuro2A cells

To study the B6 vitamer distribution within brain cells, we used Neuro2A (mouse neuroblastoma) cells as a model system, in which we investigated uptake, metabolism and excretion of vitamin B6.

Western Blot analysis showed that all three enzymes involved in vitamin B6 metabolism (PDXK, PNPO and PDXP) were present in Neuro2A cells (Figure 2).

In the absence of vitamin B6 in medium, PMP and PLP were the dominant B6 vitamers detected intracellularly (Table 1; Figure 3A).

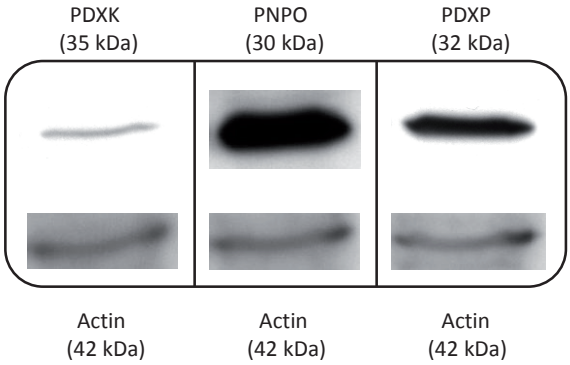

Figure 2 Expression of the enzymes involved in vitamin B6 metabolism (PDXK, PNPO and PDXP) by Neuro2A cells.
PDXK = pyridoxal kinase. PNPO = pyridox(am)ine phosphate oxidase. PDXP = vitamin B6-specific phosphatase.

Table 1 Distribution of B6 vitamers in confluent Neuro2A cells, grown in medium containing either no vitamin B6 or 20 µmol/L (40 nmol) of PN, PM or PL.
Depicted are mean B6 vitamer concentrations (pmol/mg) ± SEM (standard error of the mean). PA was not detected intracellularly in any condition.

Intracellular B6 vitamer	**PN**	**PNP**	**PL**	**PLP**	**PM**	**PMP**
No B6 in medium	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	68 ± 2.1	2.1 ± 0.4	154 ± 8.5
PN in medium	351 ± 16	20 ± 2.3	57 ± 3.1	326 ± 20	2.0 ± 0.1	275 ± 13
PM in medium	0.3 ± 0.2	0.2 ± 0.2	60 ± 2.0	243 ± 12	393 ± 11	519 ± 18
PL in medium	1.5 ± 0.2	0.0 ± 0.0	524 ± 22	204 ± 11	4.5 ± 0.3	216 ± 5.7

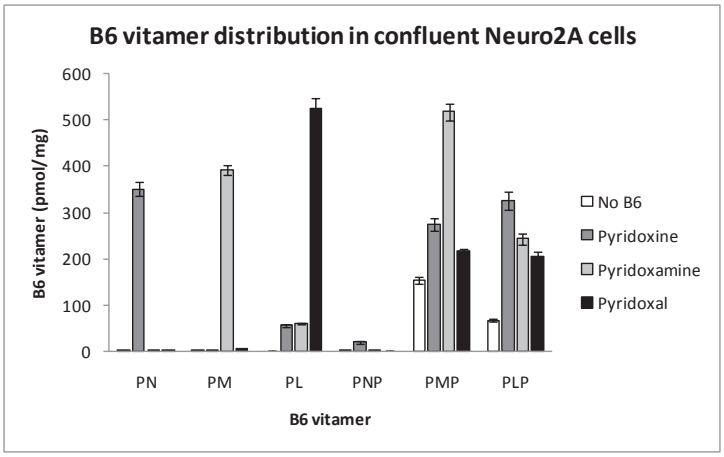

Figure 3A The B6 vitamer distribution within confluent Neuro2A cells, grown in medium containing either no vitamin B6 or 40 nmol of PN, PM or PL.
Each bar represents the mean B6 vitamer concentration (pmol/mg) ± SEM. PA was not detected intracellularly in any condition.

In the presence of PL in medium, reflecting the *in vivo* situation of PL as the dominant B6 vitamer in CSF, PL became detectable intracellularly. Intracellular concentrations of PLP and PMP increased compared to intracellular concentrations of these B6 vitamers in the absence of vitamin B6 in medium. These findings suggest that Neuro2A cells take up PL and convert it into PLP and PMP. The intracellular B6 vitamer distribution in this situation was PL>PMP~PLP (Table 1; Figure 3A).

During exposure to PM, which in lower concentrations is usually also present in CSF, the unphosphorylated B6 vitamers detected intracellularly were PM and PL. Intracellular

concentrations of PLP and PMP increased three to four times. Thus, PM is taken up from the medium and is converted into PMP, PLP and PL, resulting in an intracellular B6 vitamer distribution of PMP>PM>PLP>PL (Table 1; Figure 3A).

In the presence of PN in medium, reflecting the *in vivo* situation of supplementation since PN is normally undetectable in CSF, PN became detectable intracellularly, in addition to PNP and PL. Intracellular concentrations of PLP and PMP became almost five- and two-fold higher, respectively, than in the absence of vitamin B6 in medium. This suggests that, just like PL and PM, Neuro2A cells take up PN and convert it into PNP, PLP, PL and PMP. The intracellular B6 vitamer distribution in this situation was PN>PLP>PMP>PL>PNP (Table 1; Figure 3A).

In the presence of PM and PN in medium, Neuro2A cells not only metabolized these B6 vitamers into PLP and PL, but also excreted PL and PLP into the medium (Figure 3B). Amounts of PL were more than two times higher in PN- than in PM-containing medium, whereas intracellular concentrations of PL did not differ between these conditions. Amounts of PLP were equal in PN- and PM-containing medium, but were almost two times lower in PL-containing medium. The other phosphorylated B6 vitamers, PNP and PMP, were not detected in medium.

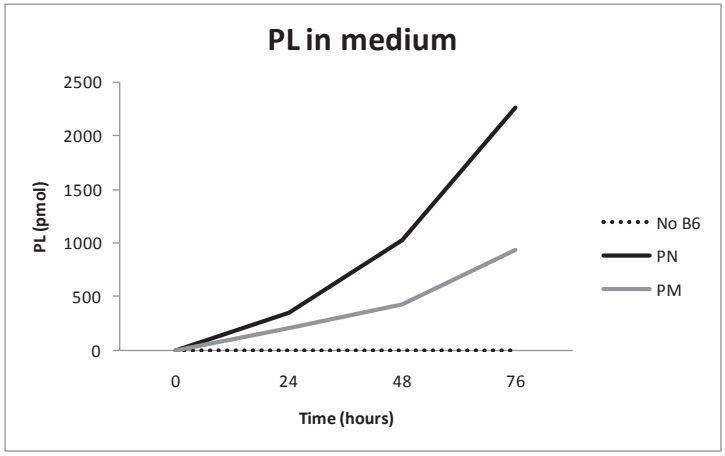

Figure 3B Cumulative amounts of PL and PLP in medium of Neuro2A cells, grown in either no vitamin B6 or 40 nmol of PN, PM or PL.

Interestingly, in the presence of PL, Neuro2A cells excreted a small amount (84 pmol) of PN while intracellular concentrations of PN were only 1.5±0.2 pmol/mg. This suggests that after uptake of PL, Neuro2A cells are able to convert PL into PN and excrete this into the medium.

PA was not detected intracellularly in any condition, but was excreted into the medium when Neuro2A cells were exposed to PL (31 pmol).

Consequences of PNPO deficiency

To study the effects of a deficiency of the PNPO enzyme on the B6 vitamer distribution within Neuro2A cells, RNA interference of the *PNPO* gene was performed after which the cells were exposed to PN.

Expression of the PNPO protein was reduced to 10, 38, 17 and 34% for siRNA 1, 2, 3 and 4, respectively (means of duplicates), compared to the expression of PNPO for control siRNA (Figure 4).

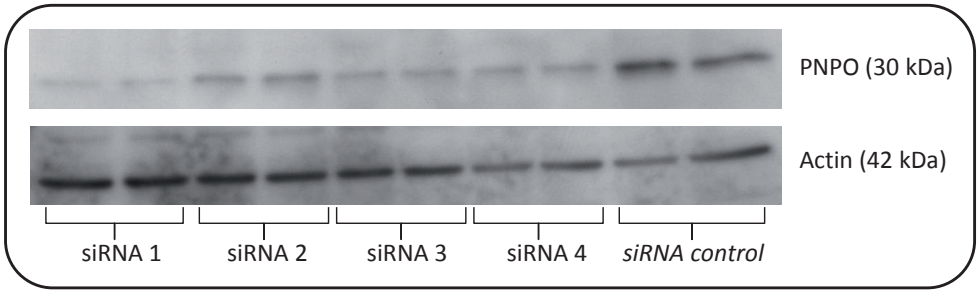

Figure 4 Expression of PNPO by Neuro2A cells after RNA interference of the *PNPO* gene.
The expression of PNPO in cells transfected with siRNA 1, 2, 3 and 4 after normalization to the expression of PNPO in cells transfected with control siRNA and to the expression of Actin, was 10, 38, 17 and 34%, respectively (means of duplicates).

From the experiments described above, we know that Neuro2A cells take up PN and convert it into PNP, PLP, PL and PMP. Exposure to PN after knockdown of PNPO with siRNA 1, 3 and 4 resulted in higher intracellular concentrations of PN compared to the control situation (Figure 5, Supplementary Table 1). Uptake of PN from the medium was equal for PNPO depleted and control cells, with the exception of siRNA 2, for which uptake of PN was lower (data not shown).

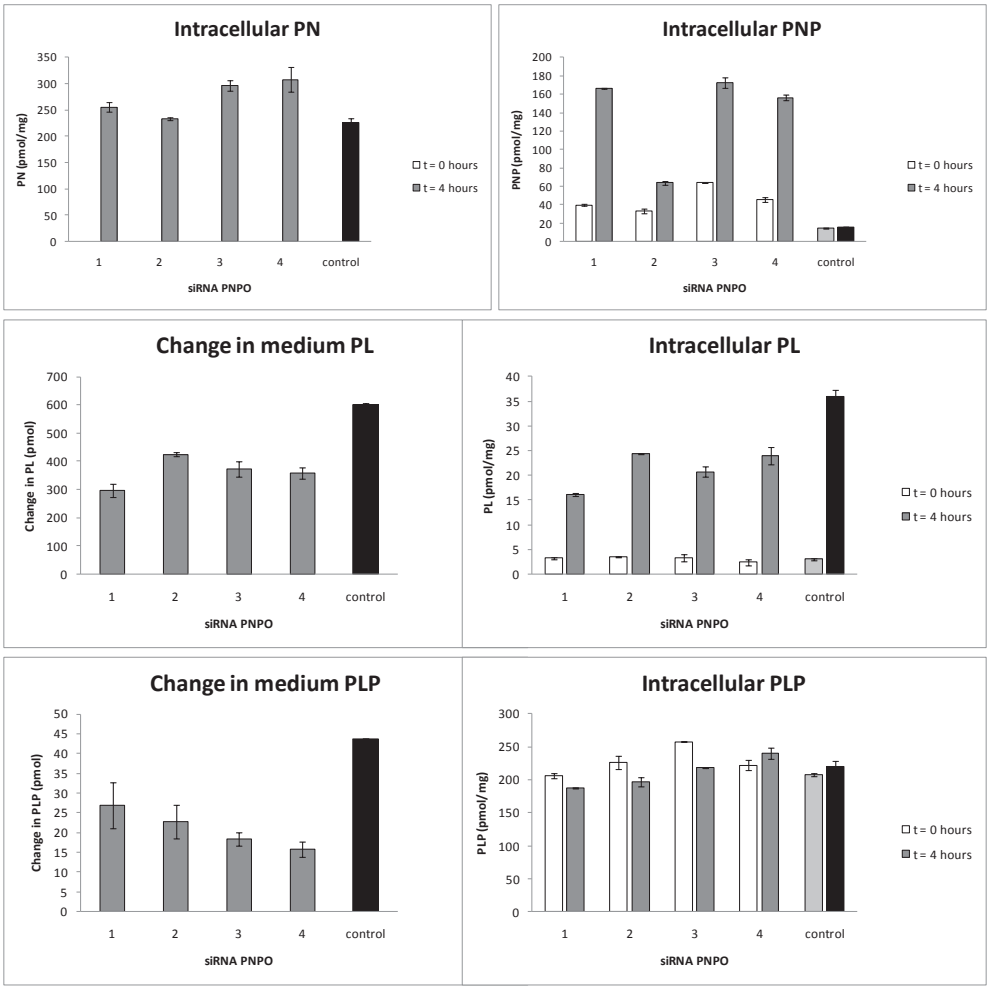

Figure 5 The B6 vitamer distribution within Neuro2A cells and the change in medium B6 vitamers after PNPO knockdown and incubation with 40 nmol of PN.
Each bar represents the mean B6 vitamer concentration (pmol/mg) with the range depicted at the end.

Intracellular concentrations of PNP were two to four times higher in PNPO depleted cells compared to control cells (Figure 5, Supplementary Table 1). During exposure to PN, concentrations of PNP did not change in control cells, but increased maximal 11-fold in PNPO depleted cells. Furthermore, PNPO depleted cells excreted trace amounts of PNP into the medium (up to 0.45±0.05 pmol), which was not observed for control cells.

Amounts of PLP excreted into the medium were substantially lower for PNPO depleted cells than for control cells (Figure 5). Intracellularly, PLP did not differ substantially between

Table 2 The effect of treatment with PN (*n*=3) or PLP (*n*=2) on B6 vitamer concentrations and ratios in CSF of human subjects.

Depicted are mean B6 vitamer concentrations and ratios after duplicate measurements in CSF (upper limits of normal values and lower limits of normal ratios [Albersen et al (2012)]).

Subject no.	R/ B6 vitamer	Dosage	Age	Diagnosis	PN (nM)	PL (nM)	PLP (nM)	PM (nM)	PA (nM)	Ratio PLP:PL	Ratio PA:PL
1	PN	100mg/day	9 d	No	**23163** (<0.03)	**1933** (<199)	91 (<221)	**12** (<3.3)	47 (<52)	*0.05* (>0.45)	*0.02* (>0.04)
2	PN	175mg/day	6 m	No	**1.1** (<0.03)	**170** (<103)	**80** (<76)	0.8 (<1.4)	7.0 (<11)	0.47 (>0.15)	0.04 (>0.02)
		500mg/day	7 m		**7970** (<0.03)	**3300** (<103)	**97** (<76)	**63** (<1.4)	**19** (<11)	*0.03* (>0.15)	*0.01* (>0.02)
3	PLP	90mg/day	1 d	No	**368** (<0.03)	**3940** (<199)	54 (<221)	**63** (<3.3)	**548** (<52)	*0.01* (>0.45)	0.14 (>0.04)
4	PLP	100mg/day	17 d	Antiquitin deficiency	**168** (<0.03)	**2078** (<103)	**129** (<76)	**19** (<1.4)	**136** (<11)	*0.06* (>0.15)	0.07 (>0.02)

Increased B6 vitamer concentrations are depicted in **bold**. Decreased B6 vitamer ratios are depicted in *italic*.

conditions (Figure 5, Supplementary Table 1). During exposure to PN, concentrations of PLP decreased in PNPO depleted cells (with the exception of siRNA 4), but increased in control cells.

Intracellular concentrations of PL did not differ between conditions, but during exposure to PN, intracellular PL increased and in PNPO depleted cells it was only 40-70% of the concentration observed in control cells (Figure 5, Supplementary Table 1). In medium,

PL appeared in all conditions, but amounts for PNPO depleted cells were only 50-70% of the amount for control cells (Figure 5).

Intracellular concentrations of PM and PMP were equal in all conditions. In medium, amounts of PM were higher for PNPO depleted cells (25±3.4 - 38±0.1 pmol) than for control cells (13±2.9 pmol). PMP was not detected in medium. PA was not detected intracellularly nor in medium in any condition.

The effect of vitamin B6 supplementation on the B6 vitamer composition of CSF

To study the effect of treatment with vitamin B6, B6 vitamer concentrations were determined in CSF of children supplemented with PN (*n*=3) or PLP (*n*=2). With PN as well as PLP supplementation, concentrations of PN, PM, PL and PA in CSF were strongly elevated compared to non-supplemented subjects (Table 2). Concentrations of PLP were at the upper limit of normal or only slightly elevated. PMP remained below the limit of quantification and PNP could not be quantified in CSF [Van der Ham et al (2012)]. The ratio between PLP and PL in CSF decreased with supplementation of both PN and PLP, whereas the ratio between PA and PL in CSF decreased only with supplementation of PN and remained normal with supplementation of PLP (Table 2).

Interestingly, PN was not only detected in high concentrations in CSF of children supplemented with PN, but also in CSF of children supplemented with PLP. To investigate the possibility of contamination of PLP with PN, we quantified the different B6 vitamers in PLP tablets (50mg) dissolved in TCA (10mL). One tablet of PLP contained only 15ng of PN (0.00003%), implicating that with PLP supplementation, PN is formed from PLP, probably through PL, after which it becomes detectable in CSF (Table 2).

DISCUSSION

We investigated uptake, metabolism and excretion of vitamin B6 by Neuro2A cells and studied the intracellular consequences of a deficiency of PNPO as well as the effects of vitamin B6 supplementation.

The B6 vitamer distribution within Neuro2A cells versus the B6 vitamer composition of CSF

We investigated the effects of vitamin B6 supplementation in an *in vitro* model system as well as in CSF of children supplemented with vitamin B6.

In CSF of non-supplemented children, PL and PLP are the dominant B6 vitamers [Albersen et al (2012)] [Albersen et al (submitted)] [Van der Ham et al (2012)]. During supplementation with PN or PLP, we observed strongly elevated concentrations of PN, PM, PL and PA in CSF, whereas concentrations of PLP were only slightly elevated or at the upper limit of normal, regardless of the supplemented B6 vitamer. This implicates that concentrations of PLP are kept relatively constant despite highly increased concentrations of the precursor B6 vitamers, and in particular PL. This is reflected by the observed decrease in the ratio between PLP and PL in CSF when children are supplemented with vitamin B6.

In our *in vitro* model of Neuro2A cells, the enzymatic system involved in vitamin B6 metabolism proved to be fully represented and we showed that the intracellular B6 vitamer distribution is dependent on the B6 vitamer composition of the medium. In the absence of extracellular vitamin B6, PMP, PLP and, to a much lesser extent, PM, were the only B6 vitamers detected intracellularly. This is in line with findings in rat brain, where PMP and PLP appeared to be the dominant B6 vitamers (PMP:PLP = ~2:1) [Sampson and O'Connor (1989)] [Sharma and Dakshinamurti (1992)]. Both PMP and PLP in rat brain decreased during vitamin B6 depletion (to 85% and 50%, respectively) [Sampson and O'Connor (1989)]. In Neuro2A cells depleted of vitamin B6, concentrations of PMP and PLP were <60% and <30%, respectively, of intracellular concentrations observed when vitamin B6 was present in the medium.

Addition of the unphosphorylated B6 vitamers to the medium resulted in uptake, and as a result, substantial concentrations of intracellular PN, PM and PL. Subsequently, phosphorylation occurred, as is illustrated by the detection of PNP and PMP intracellularly (Figure 6A). Furthermore, intracellular concentrations of PLP and PMP were higher than in the situation of vitamin B6 depletion.

Intracellular concentrations of the active cofactor PLP were the highest when PN (and not PL) was present extracellularly. This suggests that the pathway of phosphorylation of PN by PDXK, followed by oxidation of PNP by PNPO to yield PLP, is more efficient than phosphorylation of PL to yield PLP (Figure 6A). Indeed, a higher substrate affinity of PDXK for PN than for PL has been reported [Fong et al (2002)].

We did not only observe uptake and metabolism of the unphosphorylated B6 vitamers, but also excretion of PL and PLP by Neuro2A cells. Especially during exposure to PN, both PL and PLP appeared in medium, suggesting that maximal intracellular concentrations of these B6 vitamers were reached and that surplus was excreted, rather than being degraded into PA (Figure 6A). Excretion of PLP from neuronal cells may explain the presence of PLP in CSF [Albersen et al (2012)] [Albersen et al (submitted)] [Van der Ham et al (2012)]. PLP

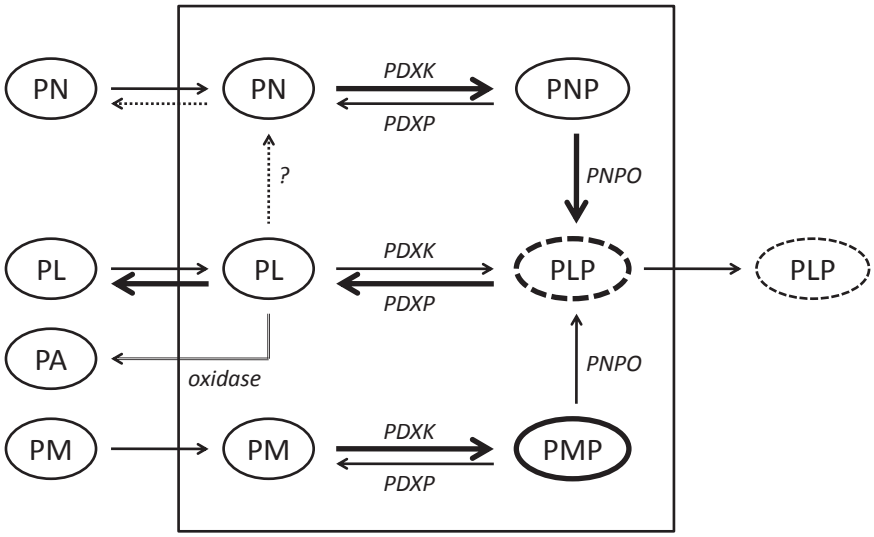

Figure 6A Model of the Neuro2A cell B6 vitamer distribution and its dependence on extracellular vitamin B6.
Shown are the uptake of the unphosphorylated B6 vitamers PN, PM and PL by Neuro2A cells. Intracellular phosphorylation by PDXK is in favor of PN and PM above PL. The resulting PNP and PMP are oxidized into PLP by the action of PNPO, which is in favor of PNP above PMP. Neuro2A cells mainly excrete PL and PLP. Only when intracellular concentrations of PL are high, Neuro2A cells excrete PN and PA, due to reduction involving a yet unknown enzyme and degradation by pyridoxal oxidase, respectively.

is not transported across the blood brain barrier as such, and only choroid plexus has been known to excrete PLP [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies]. We demonstrate *in vitro* the excretion of PLP by neuronal cells, which might be explained as a way to store PLP in CSF instead of degrading it into PA.

Neuro2A cells excreted PA only during exposure to PL and not during incubation with PN or PM (Figure 6A). This observation can be explained by degradation of excess PL into PA, through the enzymatic action of pyridoxal oxidase [Merrill et al (1984)]. Likewise, concentrations of PA in CSF were mainly elevated when PLP was supplemented.

A surprising finding was that Neuro2A cells excreted PN into the medium when intracellular concentrations of PL were high. This observation suggests that neuronal cells are able to convert PL into PN, a pathway that is not known in humans (Figure 6A). Likewise, PN, which is usually not detected in CSF, was not only very high in CSF of children supplemented with PN, but also in CSF of children supplemented with PLP. This suggests that PN not only passes the blood brain barrier and/or choroid plexus to appear in CSF, but that it can also be produced from PLP, probably through PL.

In plants (Arabidopsis Thaliana [Herrero et al (2011)]) as well as yeast (Saccharomyces Pombe [Nakano et al (1999)]), the enzyme pyridoxal reductase is known to convert PL into PN. Our observations suggest that in humans a similar enzyme is present. Alternatively, PNPO might be able to catalyze its reaction in the opposite direction, from PLP to PNP. Footitt et al (2013) have recently observed PN in plasma of children supplemented with PLP.

An intriguing phenomenon is the neurotoxicity of high doses of PN *in vivo*. Rudman and Williams [Rudman and Williams (1983)] proposed that when large amounts of PN are ingested, the livers capacity to fully metabolize this into PLP is exceeded and PN ends up in neuronal cells. As a consequence, there may be intracellular competition between PN and PL for phosphorylation to PNP and PLP, respectively. Thus, a functional deficiency of PLP might explain the neurotoxicity observed in case of PN supplementation. Our studies, showing that with PN supplementation, indeed PN can be found in CSF and showing that uptake of PN can take place in neuronal cells, are in support of this hypothesis. However, we show that neuronal cells exposed to PN have sufficient intracellular concentrations of PLP, which is in contrast with the hypothesis of Rudman and Williams. Based on our findings, we speculate that it is not a deficiency of PLP, but rather an excess of intracellular PN that causes neurotoxicity of vitamin B6 [Levine et al (2004)] [Jortner et al (2000)].

Consequences of PNPO deficiency

Taken together the effect of PNPO depletion and incubation with PN on the B6 vitamer distribution within Neuro2A cells (both intracellularly and in medium), amounts of PN and PNP were higher whereas amounts of PLP and PL were lower (Figure 6B). This implies hampered PLP formation and accumulation of PNP due to a deficiency of the PNPO enzyme. In CSF of patients suffering from PNPO deficiency, decreased concentrations of PLP [Goyal et al (2013)] [Mills et al (2005)] [Ruiz et al (2008)] and PL [Mills et al (2005)] have been found. Concentrations of the other B6 vitamers were not reported. Accumulation of PNP has been recently observed in plasma of PNPO deficient patients receiving PLP treatment [Footitt et al (2013)].

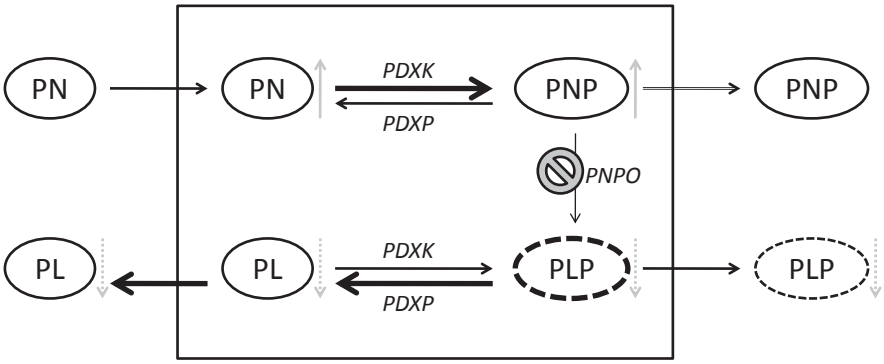

Figure 6B Model of the effects of PNPO knockdown and incubation with PN on the Neuro2A cell B6 vitamer distribution.
Shown are hampered formation and excretion of PL and PLP, as well as intracellular accumulation of PN and PNP and excretion of PNP by Neuro2A cells after knockdown of the PNPO enzyme and incubation with PN.

CONCLUSION

Our *in vivo* data on vitamin B6 in CSF show that both PN and PLP supplementation lead to supraphysiological concentrations of all but one B6 vitamer. Despite the high doses of supplemented PLP and PN, PLP concentrations in CSF remain almost within normal limits. Our *in vitro* studies suggest that these high concentrations of B6 vitamers in CSF have an intracellular effect: concentrations of the unphosphorylated B6 vitamers increase, the phosphorylated B6 vitamers appear and PA is formed. Furthermore, the effects of PN and PL on the intracellular B6 vitamer distribution differ, as PN generates more PLP than does PL. This suggests that under normal circumstances, PN supplementation may be more effective than PLP supplementation. However, the high intracellular concentrations of PN that are generated may contribute to neurotoxicity and, as expected, in PNPO deficiency PN is not effective in maintaining normal intracellular concentrations of PLP.

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SUPPLEMENTARY TABLE

Supplementary Table 1 Distribution of B6 vitamers in Neuro2A cells after PNPO knockdown and incubation with 20 µmol/L (40 nmol) of PN.
Depicted are mean B6 vitamer concentrations (pmol/mg) ± distance to range limits. t0: t=0 hours. t4: t=4 hours.

Intracellular B6 vitamer	**PN**		**PNP**		**PL**		**PLP**		**PM**		**PMP**	
	t0	**t4**	**t0**	**t4**	**t0**	**t4**	**t0**	**t4**	**t0**	**t4**	**t0**	**t4**
siRNA 1	0.0 ±0.0	256 ±9.7	40 ±0.7	167 ±0.8	3.2 ±0.2	16 ±0.3	206 ±4.0	188 ±0.8	0.5 ±0.2	0.7 ±0.2	151 ±1.7	134 ±1.6
siRNA 2	0.0 ±0.0	234 ±2.7	33 ±2.4	64 ±2.0	3.6 ±0.1	24 ±0.1	227 ±9.7	198 ±6.8	0.4 ±0.0	0.5 ±0.0	195 ±6.2	152 ±0.2
siRNA 3	0.0 ±0.0	296 ±9.9	64 ±0.1	172 ±5.8	3.3 ±0.8	21 ±1.0	258 ±0.3	218 ±0.6	0.5 ±0.0	1.1 ±0.7	190 ±2.8	154 ±5.1
siRNA 4	0.0 ±0.0	308 ±24	46 ±2.4	157 ±3.2	2.4 ±0.7	24 ±1.8	222 ±7.8	240 ±8.9	0.4 ±0.0	0.9 ±0.2	170 ±2.6	165 ±5.2
siRNA control	*0.0 ±0.0*	*226 ±8.7*	*15 ±0.8*	*16 ±0.1*	*3.0 ±0.2*	*36 ±1.3*	*208 ±2.5*	*219 ±9.0*	*0.3 ±0.0*	*0.6 ±0.1*	*178 ±0.2*	*155 ±2.5*

CHAPTER 8

General Discussion

VITAMIN B6

Over the past years, interest in vitamin B6 has increased, since its essential role in normal brain development and functioning has been recognized and specific inborn errors of metabolism resulting in functional vitamin B6 deficiency have been identified. As discussed below, the work described in this thesis contributes to the understanding of vitamin B6 metabolism and transport.

B6 vitamers in human plasma and CSF

Disorders of vitamin B6 metabolism with known or yet uncharacterized causes may be missed by investigating the secondary biochemical effects of functional vitamin B6 deficiency [Hoffmann et al (2007)] [Khayat et al (2008)] [Mercimek-Mahmutoglu et al (2013)] [Mills et al (2005)]. Direct analysis of vitamin B6 may overcome this diagnostic limitation and may increase our insight in B6 vitamer concentrations in health and disease.

B6 vitamer concentrations in human plasma have been published. Until recently, however, information on B6 vitamers in human CSF was limited and concentrations had only been reported for PLP [Footitt et al (2011)] [Ormazabal et al (2008)] [Shin et al (1984)] (Table 1).

We developed and validated a rapid, sensitive and accurate UPLC-MS/MS method for the quantification of PL(P), PM(P), PN and PA in human CSF **(Chapter 2)**. Our method requires only minimal CSF volumes and is suitable for implementation in a routine diagnostic setting. In human CSF, PL, PLP, PA and PM are present (Table 1). PMP and PN are below the limits of quantification and PNP cannot be quantified with our method. Decreased concentrations of PL and PLP can be detected with our method and biochemical effects of vitamin B6 supplementation can be monitored.

Because vitamin B6 plays a pivotal role in normal brain development and functioning, it is relevant to obtain knowledge on B6 vitamer concentrations in CSF of newborn infants. Within the scope of diagnosing functional vitamin B6 deficiency, which results in neonatal convulsions and developmental delay, we studied B6 vitamer concentrations in CSF of preterm and term newborn infants **(Chapter 3)**. Our results indicate that vitamin B6 homeostasis in brain differs between preterm and term newborn infants (Table 1). Cell counts and protein concentrations in CSF did not influence B6 vitamer concentrations, neither did the type of nutrition (breast milk (with or without fortifier), infant formula or parenteral nutrition). Since B6 vitamer concentrations in CSF of newborn infants were

Table 1 Vitamin B6 vitamer concentrations in CSF of human subjects

Reference	Population	Range of B6 vitamer in CSF (nmol/L)			
		PL	PLP	PM	PA
Shin (1984)	German control subjects (2 mo - 68 yrs; *n*=88)[a]				
	≤1 yrs (*n*=26)		5.7 - 78		
	1-68 yrs (*n*=62)		1.2 - 52		
Ormazabal et al (2008)	Spanish paediatric controls with a neurological condition (*n*=80)				
	<30 days (*n*=7)		32 - 78		
	1-12 mo (*n*=16)		24 - 87		
	1-2 yrs (*n*=18)		14 - 59		
	3-19 yrs (*n*=39)		11 - 40		
Footitt et al (2011)	British subjects with neurological symptoms (*n*=121) [2.5 – 97.5%]				
	<30 days (*n*=7)		26 - 69		
	1-12 mo (*n*=37)		14 - 92		
	1-2 yrs (*n*=28)		11 - 64		
	3-51 yrs (*n*=49)		10 - 37		
Chapter 3	Dutch newborn infants (postmenstrual age in weeks; *n*=69)				
	<30 weeks (*n*=2)[b]	238 - 333	38 - 57	4.6 - 7.4	227 - 275
	30-37 weeks (*n*=25)	46 - 226	28 - 170	0.5 - 3.6	6.0 - 73
	37-42 weeks (*n*=23)	16 - 199	19 - 221	0.3 - 3.3	1.9 - 52
	≥42 weeks (*n*=19)	14 - 103	8.0 - 76	0.3 - 1.4	0.9 - 11
Chapter 2	Dutch children with developmental delay and/or movement disorder				
	8 mo - 16 yrs (*n*=20)	15 - 43	8.8 - 42	0.1 - 0.5	0.09 - 4.1
Chapter 4	Dutch children with developmental delay and Dutch healthy adults				
	1-18 yrs (*n*=41)	16 - 56	11 - 34	0.3 - 0.9	0.0 - 3.1
	18-63 yrs (*n*=424)	14 - 79	5.3 - 49	0.0 - 1.2	0.0 - 8.7

PMP<5.4 nmol/L (LOQ). PN<0.03 nmol/L (LOQ). % = percentile.

[a] Concentration in nmol/L was calculated from an original concentration in ng/mL.

[b] In CSF of these very preterm newborn infants, PN was also present (1.7-2.8 nmol/L).

inversely associated with postmenstrual age (postnatal age corrected for duration of pregnancy) and since they were substantially higher than in CSF of children aged 1-18 years (Table 1), reference values for B6 vitamers in CSF must take postmenstrual age into account and must be established for newborn infants and children separately. The observed higher concentrations of the unphosphorylated B6 vitamers, as well as the presence of PN, in CSF of very preterm newborns (postmenstrual age <30 weeks; Table 1), may point to an immaturity of the enzymatic system involved in vitamin B6 metabolism. Literature is inconsistent regarding the time span of development of this system throughout gestation, but we hypothesize that a decreased activity of PDXK and/or an increased activity of ALPL or PDXP at a lower postmenstrual age underlie our observation.

To date, we do not know the physiological importance of each B6 vitamer nor the best way to evaluate a person's vitamin B6 status. Simultaneously studying B6 vitamer concentrations in plasma and CSF will deepen our understanding of normal human B6 vitamers and their interrelationships. We adapted our UPLC-MS/MS method for the analysis of CSF to enable the analysis of plasma and studied B6 vitamer concentrations in plasma as well as CSF of a large number of children and adults **(Chapter 4)**. We showed that the B6 vitamer composition of plasma (PLP>PA>PL) differs from that of CSF (PL>PLP>PA>PM). PM is not detectable in plasma.

Our study identified several pre-analytical factors that must be taken into account when studying B6 vitamers in body fluids. B6 vitamer concentrations in CSF are not affected by a rostrocaudal gradient, meaning that vitamin B6 can be measured in a random CSF sample. However, it must be warranted that samples are protected from light and frozen immediately after withdrawal to prevent degradation of B6 vitamers. In order to retain B6 vitamer stability, samples should be stored at -80°C until further analysis. B6 vitamer concentrations in plasma and CSF are not influenced by epilepsy. However, concentrations of mainly PL and PLP were lower in CSF of epileptic children with anti-epileptic drug treatment, meaning that these children are at risk of a deficiency of vitamin B6, which may have adverse effects on brain development and functioning. Furthermore, concentrations of PL and PLP in plasma and CSF of adults and concentrations of PL in CSF of children, were influenced by sex. Concentrations of PL and PLP in CSF of children were also, although only marginally, influenced by age.

B6 vitamer concentrations in plasma and CSF may be very useful in clinical practice regarding diagnosis and treatment of conditions associated with altered vitamin B6 metabolism, since decreased concentrations of PL and PLP can be detected in both body fluids and the biochemical effects of vitamin B6 supplementation can be monitored.

Our observation of strict ratios and strong correlations between PLP and PL in plasma and between PA and PL in CSF suggests that concentrations of these B6 vitamers are tightly regulated. Disturbances of B6 vitamer ratios in plasma and/or CSF may indicate possible deficiencies of the enzymes involved in vitamin B6 metabolism. It is therefore important to determine concentrations of all B6 vitamers when investigating possible vitamin B6 related disease. Interestingly, the correlation between PLP and PL in plasma differed between adults and children. One might hypothesize that age-related changes in the activity of the extracellular ALPL enzyme, which hydrolyzes PLP into PL, might underlie this observation, since it is known that ALPL activities in plasma are higher in children than in adults [Fleisher et al (1977)] [Schiele et al (1983)].

Genetic regulation

Possible mechanisms determining B6 vitamer concentration differences between plasma and CSF are dietary intake, metabolism and/or transport as well as yet undefined, genetic factors. To study the genetic regulation of B6 vitamers, we conducted a GWAS (genome-wide association study) of B6 vitamer concentrations and ratios in and between plasma and CSF of healthy adult subjects **(Chapter 5)**. For B6 vitamer concentrations in plasma, we found one genome-wide significant association, between PLP, which is the dominant B6 vitamer in plasma, and a SNP (single nucleotide polymorphism) located upstream of the gene encoding a long-chain fatty acid transport protein, *SLC27A6*. Although relationships between fatty acids and vitamin B6 have been reported, the mechanism by which *SLC27A6* influences vitamin B6 remains to be elucidated. No genome-wide significant associations were found for concentrations of B6 vitamers in CSF, suggesting that these are mainly regulated by other than genetic factors.

To study genetic determinants of the relationships we found between the different B6 vitamers, we tested associations for B6 vitamer ratios in plasma and CSF and found genome-wide significance only in CSF. Several SNPs upstream of *HTR7*, encoding one of the serotonin receptors, were genome-wide significantly associated with the ratio between PA and PLP in CSF. It is generally accepted that degradation of PLP into PA occurs by the enzymatic actions of a vitamin B6-specific phosphatase (hydrolysis of PLP to PL) and pyridoxal oxidase (oxidation of PL to PA). However, our study suggests that the balance between PA and PLP in CSF is, more importantly, under regulation of another genetic factor. A direct link between serotonin receptor activity and vitamin B6 metabolism has not been reported to date.

To answer the question which of the enzymes involved in vitamin B6 metabolism is or are dominant in determining B6 vitamer concentrations, SNPs in the genes encoding ALPL,

PDXK, PNPO and PDXP were studied for their association with B6 vitamer concentrations and ratios in plasma and CSF. None of these genes showed associations at a genome-wide significance level, so it is likely that other genetic factors are more important in the regulation of B6 vitamer concentrations in plasma and CSF than the enzymes so far known to be involved in vitamin B6 metabolism. The significant relationship between PLP in plasma and the ALPL enzyme, which was found in another GWAS [Hazra et al (2009)], was not confirmed in our association analyses.

Compared to other GWAS performed on vitamin B6 in body fluids, which were only published for PLP in plasma [Hazra et al (2009)] [Tanaka et al (2009)], our sample size seems relatively small. However, our study is based on a unique collection of simultaneously drawn samples of plasma and CSF from healthy volunteers, enabling direct comparison between the two body fluids. Furthermore, GWAS data have not been reported for the other B6 vitamers, besides PLP, in plasma, nor for any of the B6 vitamers in CSF. With our GWAS of B6 vitamers (PL, PLP, PM) and PA, we obtained insight in the genetic associations of B6 vitamer concentrations and ratios. The identification of several genome-wide significant SNPs at loci containing transporter and neurotransmitter receptor genes is intriguing and the mechanism by which these genes influence vitamin B6 remains to be elucidated. Besides reproducing our findings in an additional cohort of healthy human subjects, the future challenge in general lies in increasing sample sizes and thus power, as well as validity, of studies investigating genetic associations of metabolites in plasma and CSF of human subjects.

Vitamin B6 metabolism

Vitamin B6 is the term used to indicate a group of unphosphorylated and phosphorylated pyridine compounds that can be enzymatically interconverted. These interconversions are essential, since dietary sources mostly contain PN(P) and PM(P) whereas the biologically active cofactor of vitamin B6 is PLP. The organs that are important in the conversion of precursor B6 vitamers into PLP however, have not been irrefutably identified. Although it has been generally accepted to be the liver, there is a discrepancy in literature regarding the main location of vitamin B6 metabolism, because the intestine has also been implicated [Sakurai et al (1987)] [Sakurai et al (1988)] [Sakurai et al (1991)].

We investigated the role of the intestine in human vitamin B6 metabolism using polarized Caco-2 cell monolayers, which constitute an *in vitro* model of human intestinal enterocytes **(Chapter 6)**. Our results shed new light on human vitamin B6 metabolism, as we demonstrated a substantial role for the intestine. We show that PN, PM and PL are

transported from the apical compartment (a reflection of the intestinal lumen) into intestinal cells and that PN and PM are converted into PL, which is released mainly into the basolateral compartment (a reflection of the portal blood side of the intestine). These interconversions are likely due to the sequential actions of PDXK, PNPO and PDXP, since these enzymes were found to be expressed by intestinal enterocytes. Apical excretion of PA was found primarily during incubation with PL, which fits the known degradation pathway of vitamin B6 involving oxidation of PL into PA. In addition, intracellular levels of PLP were found to be within a small range and PLP was not excreted by Caco-2 cells. Furthermore, we found that when high doses of PN or PM are administered, the capacity of the intestine to fully convert these B6 vitamers into PL is exceeded and PN and PM enter the portal blood, after which they will probably be metabolized in the liver as well. Under normal dietary circumstances, it is thus very likely that not PN or PM, but mainly PL reaches the portal circulation. All other organs, including liver and brain, obtain PL from the blood and only need PDXK to form PLP. (Figure 1) This suggests that in most tissues, including the brain, which is highly dependent on PLP for proper development and functioning, PNPO is not needed for PLP formation. Instead, it might act as a recycling enzyme in the salvage pathway of PLP rather than as a PLP synthesis enzyme.

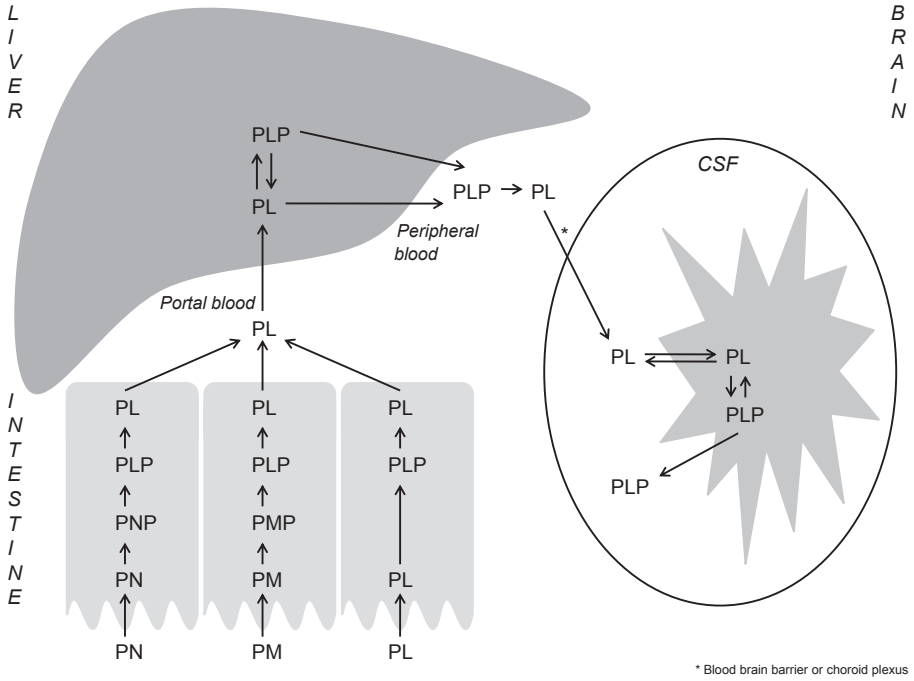

Figure 1 Model of human vitamin B6 metabolism.

To gain knowledge on vitamin B6 metabolism in the brain, we used an *in vitro* mouse model of neuronal cells: the Neuro2A cell line **(Chapter 7)**. We showed that Neuro2A cells take up PN, PM and PL and metabolize these B6 vitamers into PNP, PMP and PLP. The enzymatic system involved in vitamin B6 metabolism was fully expressed by Neuro2A cells and the intracellular B6 vitamer distribution was different for each B6 vitamer present extracellularly. In the absence of vitamin B6, PMP and PLP were the main intracellular B6 vitamers, although their amounts were much lower than observed in the presence of extracellular vitamin B6. Intracellular amounts of the active cofactor PLP were the highest when PN (and not PL) was present extracellularly. This suggests that the pathway of phosphorylation of PN by PDXK, followed by oxidation of PNP by PNPO to yield PLP, is more efficient than phosphorylation of PL to yield PLP. A higher substrate affinity of PDXK for PN than for PL has been reported in literature [Fong et al (2002)]. During incubation with PL, degradation of PL into PA was observed and, interestingly, also excretion of PN. The latter observation was surprising, since it suggests that neuronal cells are able to convert PL into PN, a pathway that is not known in humans. In plants [Herrero et al (2011)] as well as yeast [Nakano et al (1999)], the enzyme pyridoxal reductase is known to execute this conversion. Alternatively, human PNPO might be able to catalyze its reaction in the opposite direction. In CSF of children supplemented with PLP, we also observed increased concentrations of PN (see below), so the conversion of PL into PN seems to be a consistent finding.

We did not only observe uptake and metabolism of the unphosphorylated B6 vitamers by Neuro2A cells, but also excretion of PL and PLP, especially during incubation with PN. This suggests that maximal intracellular concentrations of these B6 vitamers were reached and that surplus was stored extracellularly, rather than being degraded into PA. Excretion of PLP by neuronal cells may explain the presence of PLP in CSF **(Chapter 2, 3 and 4)**. (Figure 1) PLP is not transported across the blood brain barrier as such, and only choroid plexus has been known to excrete PLP [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies].

Vitamin B6 transport

Vitamin B6 is provided by the diet, enters the human body through intestinal uptake and is metabolized by the intestine and liver. Although the enzymes involved in vitamin B6 metabolism have been elucidated at genetic and protein levels, knowledge on vitamin B6 transport is limited. At the biochemical level, there is evidence for carrier-mediated transport in the intestine [Said et al (2003)] [Said et al (2008)] as well as choroid plexus and blood brain barrier [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies] [Spector and Johanson (2007)], but a vitamin B6 transport protein has not yet been characterized.

All three unphosphorylated B6 vitamers, PN, PM and PL, were taken up by Caco-2 as well as Neuro2A cells, in the same order of magnitude **(Chapter 6 and 7)**.

Our observation that concentrations of PL are higher in CSF than in plasma **(Chapter 4)**, supports other evidence that transport of PL takes place through an active mechanism at the blood brain barrier or choroid plexus. We hypothesized that the ratio of PL between CSF and plasma might be associated with SNPs in or near the gene encoding the PL transport protein in choroid plexus and/or blood brain barrier. We detected suggestively significant associations with two SNPs in an uncharacterized locus (*LOC100506272*) on chromosome 4 **(Chapter 5)**. In order to further investigate the influence of *LOC100506272* on the ratio of PL between CSF and plasma, this association should be validated in an additional cohort of healthy human subjects. Besides this, *in vitro* cell models should be developed in which the effects of manipulation of *LOC100506272* expression can be studied.

Consequences of PNPO deficiency

Whereas decreased concentrations of PL and PLP have been reported in CSF of PNPO deficient patients [Goyal et al (2013)] [Mills et al (2005)] [Ruiz et al (2008)], the consequences of PNPO deficiency for the B6 vitamer distribution within brain cells are not known. We developed an *in vitro* Neuro2A cell model of PNPO deficiency using RNA interference of the *PNPO* gene **(Chapter 7)**. We show that knockdown of PNPO and incubation with PN not only results in hampered formation of PLP and PL, but also in a disbalance between B6 vitamers, with accumulation of PN and PNP.

Effects of vitamin B6 supplementation

In literature, ingestion of large doses of PN has been reported to be toxic and to result in polyneuropathy [Hartmann et al (2011)] [Jortner (2000)]. The mechanism of this neurotoxicity has not yet been elucidated. In CSF of non-supplemented human subjects, PL and PLP are the dominant B6 vitamers and PN is not present **(Chapter 2, 3 and 4)**. We studied the effects of large dose vitamin B6 supplementation on B6 vitamer concentrations in CSF of children **(Chapter 2 and 7)**. We observed supraphysiological concentrations of PN, PM, PL and PA in CSF when children were supplemented with PN or PLP. Concentrations of PLP in CSF remained almost within normal limits and the ratio between PLP and PL decreased. This implicates that concentrations of PLP in CSF are kept relatively constant, despite strongly elevated concentrations of the precursor B6 vitamers, in particular PL, and regardless of the supplemented B6 vitamer. In contrast, but corresponding to our

observations in Neuro2A cells, concentrations of PA in CSF were mainly elevated when PLP was given. Furthermore, the effects of PN and PL on the intracellular B6 vitamer distribution and B6 vitamer excretion of Neuro2A cells differed, as PN generated more PLP than did PL. This suggests that under normal circumstances, PN supplementation may be more effective than PLP supplementation.

PN in CSF was not only increased in case of supplementation with PN, but also in case of supplementation with PLP. This suggests that PN not only passes the blood brain barrier and/or choroid plexus to appear in CSF, but that it can also be produced from PLP, probably through PL. Indeed, we observed formation of PN *in vitro* when we incubated Neuro2A cells with PL. This pathway is not known in humans and is interesting to be explored further.

From **Chapter 6**, we know that when high amounts of PN are ingested, the capacity of the intestine is insufficient to fully metabolize all PN. As a consequence, PN may reach the circulation and other tissues, including the liver. Indeed, in plasma of subjects supplemented with PN, it was present in quantifiable amounts (unpublished observations), whereas PN is normally undetectable in plasma. The high intracellular concentrations of PN that are probably generated this way, as well as the presence of PN in CSF, may contribute to neurotoxicity.

CONCLUSION AND PERSPECTIVES

In this study, we obtained insight in B6 vitamer concentrations in plasma and CSF of healthy subjects. Unfortunately, detailed information on alterations of these concentrations in patients with functional vitamin B6 deficiency due to specific inborn errors of metabolism, is still lacking. These inborn errors are rare and pre-treatment samples are scarce. Furthermore, samples should have been stored at -80°C prior to analysis in order to obtain reliable B6 vitamer concentrations. Continuing the quantification of B6 vitamers in plasma and CSF of patients suffering from epilepsy and/or developmental delay will increase insight in the B6 vitamer composition of their body fluids. This will be useful for the understanding of the pathogenesis of vitamin B6-related disorders and will help to optimize diagnosis and treatment.

A deficiency of vitamin B6, or a disturbance in the B6 vitamer balance, leads to other, secondary biochemical abnormalities as well as clinical symptoms. In some patients, all symptoms can be eliminated by supplementation of vitamin B6, while other patients have persistent problems [Bok et al (2012)] [Stockler et al (2011)]. Insight in the pathogenesis of functional vitamin B6 deficiency is scarce as is knowledge on the biochemical effects of treatment. To study the pathogenic mechanisms of disturbances in the metabolism

of vitamin B6, we developed an *in vitro* neuronal cell model of PNPO deficiency by RNA interference of the *PNPO* gene and investigated the intracellular effects of PNPO knockdown and supplementation with vitamin B6. Currently, we are expanding these experiments by silencing the genes encoding PDXK, PDXP and antiquitin. Addition of different B6 vitamers to the medium of these manipulated cells will teach us their potential in restoring a normal intracellular B6 vitamer composition.

Next to our *in vitro* neuronal cell model, zebrafish constitute an interesting model to study vitamin B6 metabolism *in vivo*. In a recent publication, zebrafish larvae were exposed to ginkgotoxin, which resulted in seizure-like movements [Lee et al (2012)]. Addition of PLP or γ-aminobutyrate (GABA) to the fish water entirely resolved the seizure-like phenotype, suggesting that inhibition of PDXK by ginkgotoxin (a known mechanism) leads to a decrease in the inhibitory neurotransmitter GABA. In our lab, we recently generated pilot data on B6 vitamer concentrations of normal zebrafish larvae, which indicate that this model is very promising with regard to future studies.

Very striking in the field of vitamin B6 research is the scarcity of knowledge on any vitamin B6 transporter. Few publications report biochemical data on vitamin B6 transport mechanisms in the intestine [Said et al (2003)] [Said et al (2008)] and kidney [Said et al (2002)], as well as in choroid plexus and blood brain barrier [Spector (1978) – *in vivo* studies] [Spector (1978) – *in vitro* studies] [Spector and Johanson (2007)]. However, not a single vitamin B6 transporter has been identified at the genetic level. We hypothesize the existence of more than one cell membrane transporter and at least one mitochondrial transporter. In the beginning of the study described in this thesis, we put much effort into the identification of vitamin B6 transporters using yeast knockout models, however, without any success. Later on, we followed a different approach by performing a GWAS, in which we sought for common genetic variants that were associated with the ratio of PL between CSF and plasma. We speculated that a transporter in choroid plexus and/or blood brain barrier exists and that this transporter influences the ratio between CSF and plasma of the B6 vitamer that is probably transported, PL. We did find an association with an uncharacterized locus (*LOC100506272*), which we are planning to further investigate.

Only a few specific inborn errors resulting in functional vitamin B6 deficiency have been reported. However, from literature and from our own experience, we know that not all patients responding to vitamin B6 supplementation suffer from one of these defects [Kanno et al (2007)] [Schmitt et al (2010)] [Veerapandiyan et al (2011)]. By performing whole exome or whole genome sequencing in patients with a functional vitamin B6 deficiency of unknown cause, we may genetically characterize the underlying defect in the near future.

This knowledge may not only be important in clinical practice, but will also be of great value in the research field, as it may clarify the relation between the affected genes and the metabolism of vitamin B6.

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SUMMARY

Vitamin B6 is a water-soluble and for humans essential nutrient. It comprises six different vitamers: pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their phosphate-esterified forms. Humans cannot synthesize vitamin B6 and must obtain it from sources in the diet. Transport across the cell membrane is preceded by hydrolysis of the phosphorylated B6 vitamers by the membrane-bound enzyme alkaline phosphatase (ALPL). Inside the cell, the unphosphorylated B6 vitamers (PN, PM and PL) are phosphorylated by pyridoxal kinase (PDXK). Pyridoxine phosphate (PNP) and pyridoxamine phosphate (PMP) are then converted by pyridox(am)ine phosphate oxidase (PNPO) into pyridoxal phosphate (PLP), the catalytically active form of vitamin B6. Release of vitamin B6 from the cell is dependent on hydrolysis of the phosphorylated B6 vitamers by a vitamin B6-specific phosphatase (PDXP), although a few cell types have been reported to release the phosphorylated B6 vitamers as well. Oxidation of PL by pyridoxal oxidase constitutes the degradation pathway of vitamin B6, of which the major product, pyridoxic acid (PA), is excreted in urine.

PLP is well known for its cofactor function in numerous enzymatic reactions in the central nervous system, where it mainly catalyzes amino acid and neurotransmitter metabolism. PLP is important for the biosynthesis of dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), glycine and D-serine and plays an essential role in normal brain development and functioning. In addition, inverse relationships of vitamin B6 with oxidative stress, inflammation, cardiovascular disease, diabetes and cancer have been reported.

A deficiency of vitamin B6 due to malnutrition is rare, since the different B6 vitamers are abundantly present in food products. Malabsorption of B6 vitamers from the diet, endogenous and exogenous nucleophiles or drugs which influence the enzymes involved in vitamin B6 metabolism may also lead to functional vitamin B6 deficiency. Clinical consequences of vitamin B6 deficiency are serious and mainly include neurological symptoms, of which seizures are the most distinctive. In addition, low levels of plasma PLP have been associated with depression and Alzheimer's disease.

Over the past years, interest in vitamin B6 has increased, since its essential role in the brain has been recognized and specific inborn errors of metabolism resulting in functional vitamin B6 deficiency have been identified, amongst which PNPO deficiency, antiquitin deficiency, congenital hypophosphatasia and hyperprolinaemia type II. In addition to these defects, there are genetically yet uncharacterized conditions that are responsive to vitamin B6. Patients present with epilepsy and, frequently, developmental delay. The use of PN or PLP to treat functional vitamin B6 deficiency is in some cases successful but in others only

partly effective. A substantial number of affected children still suffers from developmental delay and neurological symptoms, despite early treatment with large doses.

In plasma and CSF of patients affected with PNPO and antiquitin deficiencies, changes in amino acid and neurotransmitter metabolite profiles have been found, probably as a consequence of impaired functioning of PLP-dependent enzymes involved in amino acid and neurotransmitter metabolism. However, these biochemical abnormalities are not always present and therefore, diagnosing may be difficult. In other words, disturbances of vitamin B6 metabolism may be missed by biochemical profiling of the secondary effects of functional vitamin B6 deficiency. Direct analysis of B6 vitamers may overcome this diagnostic limitation and will increase our insight in normal human vitamin B6 metabolism and transport in health and disease.

In **Chapter 1** of this thesis, the enzymes involved in vitamin B6 metabolism, the biochemical characteristics of vitamin B6 transport and the numerous functions of vitamin B6 are discussed in more detail. In addition, diagnosis, treatment and outcome of specific inborn errors of metabolism resulting in functional vitamin B6 deficiency are reviewed.

The first aim of this study was to obtain insight in B6 vitamer concentrations in plasma and CSF of healthy subjects in order to further elucidate human vitamin B6 metabolism and transport. We hypothesized that with direct analysis of vitamin B6 in body fluids, functional vitamin B6 deficiency can be reliably diagnosed and the biochemical effects of supplementation with PLP and PN can be monitored. This will contribute to overcoming the limitations currently hampering optimal diagnosis and treatment of disorders underlying functional vitamin B6 deficiency. Although in human plasma, B6 vitamer concentrations had been reported, knowledge on B6 vitamer concentrations in human CSF was limited. We therefore developed and validated a rapid, sensitive and accurate UPLC-MS/MS (ultra performance liquid chromatography-tandem mass spectrometry) method for the simultaneous quantification of PL(P), PM(P), PN and PA in human CSF **(Chapter 2)**. Our method requires only minimal CSF volumes and is suitable for implementation in a routine diagnostic setting. In human CSF, PL, PLP, PA and PM are present. PMP and PN are below the limits of quantification and PNP cannot be quantified with our method. Our study identified several pre-analytical factors that must be taken into account when studying B6 vitamers in body fluids. B6 vitamer concentrations in CSF are not affected by a rostrocaudal gradient, meaning that vitamin B6 can be measured in a random CSF sample. However, it must be warranted that samples are protected from light and frozen immediately after withdrawal to prevent degradation of B6 vitamers. In order to guarantee B6 vitamer stability, samples should be stored at -80°C until further analysis.

Because vitamin B6 plays a pivotal role in normal brain development and functioning, it is relevant to obtain knowledge on B6 vitamer concentrations in CSF of newborn infants. Within the scope of diagnosing functional vitamin B6 deficiency in newborn infants, we studied B6 vitamer concentrations in CSF of preterm (born <37 weeks of gestation) and term (born between 37 and 42 weeks of gestation) newborn infants **(Chapter 3)**. Our results indicate that vitamin B6 homeostasis in brain differs between preterm and term newborn infants. Cell counts and protein concentrations in CSF did not influence B6 vitamer concentrations, neither did the type of nutrition (breast milk (with or without fortifier), infant formula or parenteral nutrition). Since B6 vitamer concentrations in CSF of newborn infants were inversely associated with postmenstrual age (postnatal age corrected for duration of pregnancy) and since they were substantially higher than in CSF of children aged 1-18 years, reference values for B6 vitamers in CSF must take postmenstrual age into account and must be established for newborn infants and children separately. The observed higher concentrations of the unphosphorylated B6 vitamers, as well as the presence of PN, in CSF of very preterm newborns (postmenstrual age <30 weeks), may point to an immaturity of the enzymatic system involved in vitamin B6 metabolism.

To date, we do not know the physiological importance of each B6 vitamer nor the best way to evaluate a person's vitamin B6 status. Simultaneously studying B6 vitamer concentrations in plasma and CSF will deepen our understanding of normal human B6 vitamers and their interrelationships. We therefore adapted our UPLC-MS/MS method for the analysis of CSF to enable the analysis of plasma and in **Chapter 4**, we studied B6 vitamer concentrations in plasma as well as CSF of a large number of children and adults. In this way, we were able to directly compare the B6 vitamer composition of both body fluids and draw conclusions regarding the relationships for the different B6 vitamers *in* as well as *between* plasma and CSF. We show that the B6 vitamer composition of plasma (PLP>PA>PL) differs from that of CSF (PL>PLP>PA>PM). B6 vitamer concentrations in plasma and CSF are not influenced by epilepsy. However, concentrations of mainly PL and PLP were lower in CSF of epileptic children with anti-epileptic drug treatment, meaning that these children are at risk of a deficiency of vitamin B6. This may have adverse effects on brain development and functioning. Furthermore, concentrations of certain B6 vitamers in plasma and CSF were influenced by sex and age. Our observation of strong correlations between PLP and PL in plasma and between PA and PL in CSF suggests that concentrations of these B6 vitamers are tightly regulated. Eventually, disturbances in these relationships may be used for identification of possible deficiencies of the enzymes involved in vitamin B6 metabolism or may point towards a problem in vitamin B6 transport. It is therefore important to determine

concentrations of all B6 vitamers when investigating possible vitamin B6-related disease. Information on B6 vitamer concentrations in plasma and CSF may be very useful in clinical practice regarding diagnosis and treatment of conditions associated with altered vitamin B6 metabolism, since decreased concentrations of PL and PLP can be detected in both body fluids and the biochemical effects of vitamin B6 supplementation can be monitored.

The B6 vitamer composition of plasma and CSF is possibly influenced by dietary intake, metabolism, transport, as well as yet undefined, genetic factors. Although the enzymes involved in vitamin B6 metabolism have been elucidated at genetic and protein levels, knowledge on vitamin B6 transport is limited. At the biochemical level, there is evidence for carrier-mediated transport in the intestine as well as choroid plexus and blood brain barrier, but a vitamin B6 transporter protein has not yet been characterized. To deepen our understanding of the genetic regulation of B6 vitamers, we conducted a GWAS (genome-wide association study) of B6 vitamer concentrations and ratios in and between plasma and CSF of healthy adult subjects and studied the association with common genetic variants **(Chapter 5)**. The identification of several genome-wide significant SNPs (single nucleotide polymorphisms) at loci containing transporter and neurotransmitter receptor genes is intriguing and the mechanism by which these genes influence vitamin B6 remains to be elucidated.

Enzymatic interconversions of B6 vitamers are essential, since dietary sources mostly contain PN(P) and PM(P) whereas the biologically active cofactor of vitamin B6 is PLP. The organs that are important in the conversion of precursor B6 vitamers into PLP however, have not been irrefutably identified. Although it has been generally accepted to be the liver, there is a discrepancy in literature regarding the main location of vitamin B6 metabolism, because the intestine has also been implicated. We therefore investigated the role of the intestine in human vitamin B6 metabolism using an *in vitro* model for intestinal enterocytes (polarized Caco-2 cell monolayers) **(Chapter 6)**. Our results shed new light on human vitamin B6 metabolism, as we demonstrated a substantial role for the intestine. We show that PN, PM and PL are transported from the apical compartment (a reflection of the intestinal lumen) into intestinal cells and that PN and PM are converted into PL, which is released mainly into the basolateral compartment (a reflection of the portal blood side of the intestine). These interconversions are likely due to the sequential actions of PDXK, PNPO and PDXP, since these enzymes were found to be expressed by intestinal enterocytes. Apical excretion of PA was found primarily during incubation with PL, which fits the known degradation pathway of vitamin B6 involving oxidation of PL into PA. In addition, intracellular levels of PLP were found to be within a small range and PLP was not excreted by Caco-2 cells. Furthermore, we

found that when high doses of PN or PM are administered, the capacity of the intestine to fully convert these B6 vitamers into PL is exceeded and PN and PM enter the portal blood, after which they will probably be metabolized in the liver as well. Under normal dietary circumstances, it is thus very likely that not PN or PM, but mainly PL reaches the portal circulation. All other organs, including liver and brain, obtain PL from the blood and only need PDXK to form PLP. This suggests that in most tissues, including the brain, which is highly dependent on PLP for proper development and functioning, PNPO is not needed for PLP formation. Instead, it might act as a recycling enzyme in the salvage pathway of PLP rather than as a PLP synthesis enzyme.

Although in **Chapter 2, 3 and 4** we have shown concentrations of the different B6 vitamers in plasma and CSF, neither the normal B6 vitamer distribution within brain cells nor the intracellular consequences of functional vitamin B6 deficiency and vitamin B6 supplementation are known, while PN-related neurotoxicity has been reported in literature. Whereas decreased concentrations of PL and PLP have been found in CSF of PNPO deficient patients, the consequences of PNPO deficiency for the B6 vitamer distribution within brain cells are not known. Therefore, in **Chapter 7** we investigated vitamin B6 metabolism in an *in vitro* mouse model for neuronal cells (the Neuro2A cell line). In addition, we studied the effects of PNPO knockdown and supplementation with PN and PLP. We show that Neuro2A cells take up PN, PM and PL and convert these B6 vitamers into PNP, PMP and PLP. In the absence of extracellular vitamin B6, PMP and PLP were the main intracellular B6 vitamers. Intracellular amounts of the active cofactor PLP were the highest when PN (and not PL) was present extracellularly. This suggests that the pathway of phosphorylation of PN by PDXK, followed by oxidation of PNP by PNPO to yield PLP, is more efficient than phosphorylation of PL to yield PLP, and that under normal circumstances, PN supplementation may be more effective than PLP supplementation. During incubation with PL, degradation of PL into PA was observed and, remarkably, also excretion of PN. The latter observation was surprising, since it suggests that neuronal cells are able to convert PL into PN, a pathway that is not known in humans. Alternatively, PNPO might be able to catalyze its reaction in the opposite direction. In CSF of children supplemented with PN or PLP, we observed supraphysiological concentrations of PN, PM, PL and PA, whereas concentrations of PLP remained almost within normal limits. This implicates that the concentration of PLP in CSF is kept relatively constant, despite strongly elevated concentrations of the precursor B6 vitamers, in particular PL, and regardless of the supplemented B6 vitamer. Because we observed increased concentrations of PN in CSF of children supplemented with PLP, the conversion of PL into PN seems to be a consistent finding. We did not only observe uptake and conversion of the unphosphorylated

B6 vitamers by Neuro2A cells, but also excretion of PL and PLP, especially during incubation with PN. This suggests that maximal intracellular concentrations of these B6 vitamers were reached and that surplus was stored extracellularly, rather than being degraded into PA. Excretion of PLP by neuronal cells may explain the presence of PLP in CSF. Furthermore, knockdown of PNPO and incubation with PN not only resulted in hampered formation of PLP and PL, but also in a disbalance between B6 vitamers, with accumulation of PN and PNP.

In this study, we obtained insight in B6 vitamer concentrations in plasma and CSF of healthy subjects **(Chapter 2, 3 and 4)**, we deepened our understanding of the genetic regulation of B6 vitamers **(Chapter 5)**, we demonstrated a role for the intestine in human vitamin B6 metabolism **(Chapter 6)** and we studied uptake, conversion and excretion of B6 vitamers by neuronal cells **(Chapter 7)**. In **Chapter 8**, our findings are discussed and our perspectives are outlined.

SAMENVATTING

Vitamine B6 is een wateroplosbare en voor de mens essentiële voedingsstof. Het bestaat uit zes verschillende vitameren (vormen): pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) en hun fosfaatesters. Het menselijk lichaam is niet in staat om vitamine B6 te maken en moet het dus opnemen uit de voeding. Transport over de celmembraan wordt voorafgegaan door hydrolyse van de gefosforyleerde B6 vitameren door het membraangebonden enzym alkalisch fosfatase (ALPL). In de cel worden de ongefosforyleerde B6 vitameren (PN, PM en PL) gefosforyleerd door pyridoxal kinase (PDXK). Pyridoxine fosfaat (PNP) en pyridoxamine fosfaat (PMP) worden vervolgens door pyridox(am)ine fosfaat oxidase (PNPO) omgezet in pyridoxal fosfaat (PLP), de katalytisch actieve vorm van vitamine B6. Uitscheiding van vitamine B6 door de cel is afhankelijk van hydrolyse van de gefosforyleerde B6 vitameren door een vitamine B6-specifieke fosfatase (PDXP). Van een aantal celtypen is beschreven dat deze ook de gefosforyleerde B6 vitameren uitscheiden. Oxidatie van PL door pyridoxal oxidase vormt de afbraakroute van vitamine B6, waarvan het hoofdproduct, pyridoxinezuur (PA), wordt uitgescheiden in de urine.

PLP staat bekend om zijn cofactor-functie in tal van enzymatische reacties in het centrale zenuwstelsel, waar het voornamelijk de stofwisseling van aminozuren en neurotransmitters katalyseert. PLP is belangrijk voor de biosynthese van dopamine, serotonine, glutamaat, γ-aminoboterzuur (GABA), glycine en D-serine en speelt een essentiële rol in de normale ontwikkeling en het functioneren van de hersenen. Tevens is er een verband beschreven tussen vitamine B6 en oxidatieve stress, inflammatie, cardiovasculaire aandoeningen, diabetes en kanker.

Een tekort aan vitamine B6 ten gevolge van ondervoeding is zeldzaam, aangezien de verschillende B6 vitameren ruimschoots aanwezig zijn in de voeding. Malabsorptie van B6 vitameren uit de voeding, endogene en exogene nucleofielen of medicatie die de enzymen die betrokken zijn bij de stofwisseling van vitamine B6 beïnvloedt, kunnen ook een functioneel tekort aan vitamine B6 tot gevolg hebben. De klinische gevolgen van een tekort aan vitamine B6 zijn ernstig en omvatten hoofdzakelijk neurologische symptomen, waarvan epileptische aanvallen het meest kenmerkend zijn. Voorts worden lage hoeveelheden PLP in plasma in verband gebracht met depressie en de ziekte van Alzheimer.

De laatste jaren is de interesse in vitamine B6 toegenomen, omdat zijn essentiële rol in de hersenen duidelijk is geworden en omdat er specifieke aangeboren stoornissen in de stofwisseling zijn geïdentificeerd die leiden tot een functioneel tekort aan vitamine B6. Hieronder vallen PNPO deficiëntie, antiquitine deficiëntie, congenitale hypofosfatemie en hyperprolinemie type II. Ook zijn er genetisch nog niet in kaart gebrachte aandoeningen die

reageren op toediening van vitamine B6. Patiënten presenteren zich over het algemeen met epilepsie en vaak ook met een ontwikkelingsachterstand. Het gebruik van PN of PLP om een functioneel tekort aan vitamine B6 te behandelen is in sommige gevallen succesvol, terwijl dit in andere gevallen slechts gedeeltelijk effectief blijkt te zijn. Een aanzienlijk deel van de aangedane kinderen heeft nog steeds een ontwikkelingsachterstand en neurologische symptomen, ondanks een vroege behandeling met hoge doseringen.

In plasma en cerebrospinale vloeistof (CSF) van patiënten met PNPO en antiquitine deficiënties zijn veranderingen in aminozuur- en neurotransmitterprofielen gevonden, waarschijnlijk ten gevolge van een verminderde functie van de PLP-afhankelijke enzymen die een rol spelen in de stofwisseling van aminozuren en neurotransmitters. Echter, deze biochemische afwijkingen zijn niet altijd aanwezig en daardoor kan het stellen van een diagnose lastig zijn. Met andere woorden, stoornissen in de stofwisseling van vitamine B6 kunnen worden gemist wanneer alleen de secundaire effecten van een functioneel vitamine B6 tekort biochemisch in kaart worden gebracht. Het direct analyseren van de B6 vitameren kan deze diagnostische beperking omzeilen en zal ons inzicht in de normale stofwisseling en het transport van vitamine B6 in de mens in gezondheid en ziekte vergroten.

In **Hoofdstuk 1** van dit proefschrift worden de enzymen die betrokken zijn bij de stofwisseling van vitamine B6, de biochemische kenmerken van vitamine B6 transport alsmede de talrijke functies van vitamine B6 in meer detail besproken. Tevens worden diagnostiek, behandeling en uitkomst van specifieke aangeboren stoornissen in de stofwisseling die leiden tot een functioneel tekort aan vitamine B6 uiteengezet.

Het eerste doel van deze studie was het verkrijgen van inzicht in concentraties van de B6 vitameren in plasma en CSF van gezonde personen om de stofwisseling en het transport van vitamine B6 in de mens verder op te helderen. Wij veronderstelden dat, door middel van het direct analyseren van vitamine B6 in lichaamsvloeistoffen, een functioneel tekort aan vitamine B6 betrouwbaar kan worden vastgesteld en de biochemische effecten van behandeling met PLP en PN kunnen worden geobserveerd. Dit zal bijdragen aan het verbeteren van de diagnostiek en behandeling van aandoeningen die ten grondslag liggen aan een functioneel vitamine B6 tekort. Ook al waren in het verleden B6 vitameer concentraties in humaan plasma beschreven, was er slechts beperkte informatie over B6 vitameer concentraties in humaan CSF beschikbaar. Daarom hebben wij een snelle, gevoelige en betrouwbare UPLC-MS/MS (ultra performance liquid chromatography-tandem mass spectrometry) methode ontwikkeld en gevalideerd om tegelijkertijd PL(P), PM(P), PN en PA in humaan CSF te kwantificeren **(Hoofdstuk 2)**. Onze methode vereist slechts minimale hoeveelheden CSF en is geschikt voor toepassing in een routine diagnostische

setting. In CSF zijn PL, PLP, PA en PM aanwezig. Concentraties van PMP en PN zijn lager dan de kwantificatielimiet en PNP kan met onze methode niet worden gekwantificeerd. Onze studie heeft diverse pre-analytische factoren geïdentificeerd waarmee rekening moet worden gehouden wanneer B6 vitameren in lichaamsvloeistoffen worden bestudeerd. B6 vitameer concentraties in CSF worden niet beïnvloed door een rostrocaudale (van boven naar beneden lopende) gradiënt, wat betekent dat vitamine B6 kan worden gemeten in een willekeurig CSF monster. Echter, er moet op worden toegezien dat monsters worden beschermd tegen licht en dat ze meteen na afname worden ingevroren om afbraak van B6 vitameren te voorkomen. Om de stabiliteit van B6 vitameren in CSF te garanderen, moeten de monsters tot aan analyse bij een temperatuur van -80°C worden opgeslagen.

Omdat vitamine B6 een onmiskenbare rol speelt in de normale ontwikkeling en het functioneren van de hersenen, is het relevant om kennis te verkrijgen over B6 vitameer concentraties in CSF van pasgeboren kinderen. Om een vitamine B6 tekort bij pasgeborenen goed te kunnen diagnosticeren, hebben wij B6 vitameer concentraties in CSF van premature (<37 weken zwangerschapsduur) en à terme (tussen 37 en 42 weken zwangerschapsduur) geboren kinderen bestudeerd **(Hoofdstuk 3)**. Onze resultaten laten zien dat de vitamine B6-huishouding in de hersenen verschilt tussen premature en à terme pasgeborenen. Celaantallen en de hoeveelheid eiwit in CSF hadden geen invloed op B6 vitameer concentraties, evenals het type voeding (borstvoeding (al dan niet gefortificeerd), flesvoeding of parenterale voeding). Omdat de B6 vitameer concentraties in CSF van pasgeborenen omgekeerd geassocieerd bleken met de 'postmenstruele leeftijd' (de postnatale leeftijd gecorrigeerd voor de zwangerschapsduur) en omdat deze beduidend hoger waren dan in CSF van kinderen in de leeftijd van 1 tot 18 jaar, moet bij het vaststellen van referentiewaarden voor B6 vitameren in CSF rekening worden gehouden met de postmenstruele leeftijd en moeten er aparte referentiewaarden komen voor pasgeborenen en oudere kinderen. De waargenomen hogere concentraties ongefosforyleerde B6 vitameren alsmede de aanwezigheid van PN in CSF van zeer premature pasgeborenen (postmenstruele leeftijd <30 weken), zou kunnen wijzen op immaturiteit van het enzymsysteem dat betrokken is bij de stofwisseling van vitamine B6.

Op dit moment kennen wij niet het fysiologische belang van iedere individuele B6 vitameer noch de beste manier om de vitamine B6 status van een persoon na te gaan. Het tegelijkertijd bestuderen van B6 vitameer concentraties in plasma en CSF zal ons begrip van de normale humane B6 vitameren alsmede hun onderlinge relaties doen toenemen. Om deze reden hebben wij onze UPLC-MS/MS methode voor de analyse van CSF aangepast om de analyse van plasma mogelijk te maken en in **Hoofdstuk 4** hebben wij

B6 vitameer concentraties in plasma en CSF van een groot aantal kinderen en volwassenen bestudeerd. Op deze manier waren wij in staat om de B6 vitameer samenstelling van beide lichaamsvloeistoffen direct met elkaar te vergelijken en conclusies te trekken wat betreft de relaties voor de verschillende B6 vitameren *in* zowel als *tussen* plasma en CSF. Wij laten zien dat de B6 vitameer samenstelling van plasma (PLP>PA>PL) verschilt van die van CSF (PL>PLP>PA>PM). B6 vitameer concentraties in plasma en CSF worden niet beïnvloed door het optreden van epilepsie. Concentraties van met name PL en PLP waren wel lager in CSF van epileptische kinderen die werden behandeld met anti-epileptische medicatie, wat betekent dat deze kinderen risico lopen op een tekort aan vitamine B6. Dit kan nadelige effecten hebben op de ontwikkeling en het functioneren van de hersenen. Daarnaast werden de concentraties van bepaalde B6 vitameren in plasma en CSF beïnvloed door leeftijd en geslacht. Onze observatie dat er sterke correlaties zijn tussen PLP en PL in plasma en tussen PA en PL in CSF suggereert dat de concentraties van deze B6 vitameren strak worden gereguleerd. Mogelijk kunnen verstoringen in deze relaties worden gebruikt voor de identificatie van deficiënties van de enzymen die betrokken zijn bij de stofwisseling van vitamine B6 of kunnen deze wijzen op een probleem in het transport van vitamine B6. Het is daarom van belang om concentraties van alle B6 vitameren te bepalen wanneer onderzoek wordt gedaan naar een mogelijk vitamine B6-gerelateerde aandoening. Informatie over B6 vitameer concentraties in plasma en CSF kan in de klinische praktijk heel nuttig zijn voor de diagnostiek en behandeling van ziekten geassocieerd met een veranderde vitamine B6 stofwisseling, aangezien verlaagde hoeveelheden PL en PLP kunnen worden gedetecteerd in beide lichaamsvloeistoffen en de biochemische effecten van behandeling met vitamine B6 kunnen worden geobserveerd.

De B6 vitameer samenstelling van plasma en CSF wordt mogelijk beïnvloed door inname vanuit de voeding, door stofwisseling, transport en door nog niet gedefinieerde, genetische factoren. De enzymen betrokken bij de stofwisseling van vitamine B6 zijn op zowel genetisch als eiwitniveau opgehelderd, maar de kennis wat betreft vitamine B6 transport is slechts beperkt. Op biochemisch niveau is er bewijs voor 'carrier'-gemedieerd transport in de darm alsmede in de plexus choroïdeus en bloed-hersenbarrière, maar een vitamine B6 transporteiwit is tot op heden niet gekarakteriseerd. Om ons begrip van de genetische regulatie van B6 vitameren te vergroten, hebben wij een GWAS (genoom-wijde associatie studie) van B6 vitameer concentraties en ratio's in en tussen plasma en CSF van gezonde volwassen personen uitgevoerd en daarin de associatie met veelvoorkomende genetische varianten bestudeerd **(Hoofdstuk 5)**. De identificatie van diverse genoom-wijd significante SNP's (enkel-nucleotide polymorfismen) op loci die transporter en neurotransmitter

receptor genen bevatten, is intrigerend en het mechanisme waarmee deze genen invloed hebben op vitamine B6 dient nog te worden opgehelderd.

Enzymatische omzettingen van B6 vitameren zijn essentieel, aangezien de voeding met name PN(P) en PM(P) bevat terwijl de biologisch actieve cofactor van vitamine B6 PLP is. De organen die belangrijk zijn in de omzetting van precursor B6 vitameren in PLP zijn echter niet ontegenzeglijk vastgesteld. Ook al is het algemeen geaccepteerd dat de lever een belangrijke locatie is voor de stofwisseling van vitamine B6, bestond er een discrepantie in de literatuur wat betreft de rol van de darm. Wij hebben daarom de rol van de darm in de humane stofwisseling van vitamine B6 onderzocht, gebruik makend van een *in vitro* model voor intestinale enterocyten (Caco-2 cellen) **(Hoofdstuk 6)**. Onze resultaten werpen een nieuw licht op de humane stofwisseling van vitamine B6, omdat wij een aanzienlijke rol voor de darm hierin aantonen. Wij laten zien dat PN, PM en PL vanuit het apicale compartiment (een reflectie van het intestinale lumen) de darmcel in worden getransporteerd en dat PN en PM worden omgezet in PL, dat met name in het basolaterale compartiment (een reflectie van de portale bloedzijde van de darm) wordt uitgescheiden. Deze omzettingen vinden waarschijnlijk plaats door de opeenvolgende acties van PDXK, PNPO en PDXP, aangezien deze enzymen door intestinale enterocyten tot expressie worden gebracht. Apicale uitscheiding van PA werd met name gezien tijdens incubatie met PL, een bevinding die past in de bekende afbraakroute van vitamine B6, waarvan oxidatie van PL tot PA een onderdeel uitmaakt. Daarnaast werden de intracellulaire hoeveelheden van PLP relatief constant gehouden en werd PLP niet door Caco-2 cellen uitgescheiden. Verder vonden wij dat wanneer grote hoeveelheden van PN of PM worden toegediend, de capaciteit van de darm om deze B6 vitameren volledig om te zetten in PL overschreden wordt en PN en PM in het portale bloed worden uitgescheiden, waarna ze waarschijnlijk tevens door de lever worden gemetaboliseerd. Onder normale omstandigheden is het dus zeer waarschijnlijk dat niet PN of PM, maar voornamelijk PL in het portale bloed terechtkomt. Alle andere organen, waaronder de lever en de hersenen, verkrijgen PL vanuit het bloed en hebben alleen PDXK nodig om hieruit PLP te vormen. Dit suggereert dat in de meeste weefsels, waaronder de hersenen, die uiterst afhankelijk zijn van PLP voor een goede ontwikkeling en een goed functioneren, PNPO niet nodig is voor de vorming van PLP. In plaats daarvan zou PNPO kunnen werken als een enzym in de terugwinning van PLP.

In **Hoofdstuk 2, 3 en 4** hebben wij concentraties van de verschillende B6 vitameren in plasma en CSF laten zien, maar de normale verdeling van B6 vitameren in hersencellen noch de intracellulaire gevolgen van een functioneel vitamine B6 tekort alsmede behandeling met vitamine B6 zijn bekend, terwijl PN-gerelateerde neurotoxiciteit in de literatuur is

beschreven. Ook al zijn verlaagde hoeveelheden PL en PLP gevonden in CSF van PNPO deficiënte patiënten, de gevolgen van een PNPO deficiëntie voor de B6 vitameer verdeling in hersencellen zijn niet bekend. Daarom hebben wij in **Hoofdstuk 7** de stofwisseling van vitamine B6 bestudeerd in een *in vitro* muismodel voor neuronale cellen (Neuro2A cellen). Daarnaast hebben wij de effecten van PNPO 'knockdown' en suppletie met PN en PLP bestudeerd. Wij laten zien dat Neuro2A cellen PN, PM en PL opnemen en deze B6 vitameren omzetten in PNP, PMP en PLP. In de afwezigheid van extracellulair vitamine B6 waren PMP en PLP de hoofdzakelijk aanwezige intracellulaire B6 vitameren. Intracellulaire hoeveelheden van de actieve cofactor PLP waren het grootst wanneer PN (en niet PL) extracellulair aanwezig was. Dit suggereert dat de route van fosforylatie van PN door PDXK, gevolgd door oxidatie van PNP door PNPO om PLP te verkrijgen, efficiënter is dan fosforylatie van PL om PLP te verkrijgen en dat, onder normale omstandigheden, suppletie met PN wellicht effectiever is dan suppletie met PLP. Tijdens incubatie met PL werd afbraak van PL naar PA gezien en, heel opmerkelijk, ook uitscheiding van PN. Laatstgenoemde observatie was verrassend, aangezien dit suggereert dat neuronale cellen in staat zijn om PL om te zetten in PN, een route die vooralsnog niet bekend is in de mens. Anderzijds zou PNPO in staat kunnen zijn om zijn reactie in tegenovergestelde richting te katalyseren. In CSF van kinderen die werden gesuppleerd met PN of PLP, zagen wij suprafysiologische concentraties van PN, PM, PL en PA, terwijl concentraties van PLP grotendeels binnen de normale grenzen bleven. Dit impliceert dat concentratie van PLP in CSF relatief constant wordt gehouden, ondanks sterk verhoogde concentraties van de precursor B6 vitameren, en dan met name PL, en onafhankelijk van de B6 vitameer waarmee wordt gesuppleerd. Omdat wij verhoogde concentraties van PN vonden in CSF van kinderen die werden gesuppleerd met PLP, lijkt de omzetting van PL in PN een consistente bevinding. Wij vonden niet alleen opname en omzetting van de ongefosforyleerde B6 vitameren door Neuro2A cellen, maar ook uitscheiding van PL en PLP, voornamelijk tijdens incubatie met PN. Dit suggereert dat maximale intracellulaire concentraties van deze B6 vitameren werden bereikt en dat surplus extracellulair werd opgeslagen, in plaats van te worden afgebroken tot PA. Uitscheiding van PLP door neuronale cellen zou de aanwezigheid van PLP in CSF kunnen verklaren. Voorts leidde 'knockdown' van PNPO en incubatie met PN niet alleen tot een belemmerde vorming van PLP en PL, maar ook tot een disbalans tussen de B6 vitameren met een opeenhoping van PN en PNP.

In deze studie hebben wij inzicht verkregen in B6 vitameer concentraties in plasma en CSF van gezonde personen **(Hoofdstuk 2, 3 en 4)**, hebben wij ons begrip van de genetische regulatie van B6 vitameren vergroot **(Hoofdstuk 5)**, hebben wij een rol voor de darm in

de stofwisseling van vitamine B6 aangetoond **(Hoofdstuk 6)** en hebben wij de opname, omzetting en uitscheiding van B6 vitameren door neuronale cellen bestudeerd **(Hoofdstuk 7)**. In **Hoofdstuk 8** worden onze bevindingen bediscussieerd en onze toekomstplannen uiteengezet.

LIST OF ABBREVIATIONS

ALPL	alkaline phosphatase
CSF	cerebrospinal fluid
PA	pyridoxic acid
PDXK	pyridoxal kinase
PDXP	vitamin B6-specific phosphatase
PL	pyridoxal
PLP	pyridoxal phosphate
PM	pyridoxamine
PMP	pyridoxamine phosphate
PN	pyridoxine
PNP	pyridoxine phosphate
PNPO	pyridox(am)ine phosphate oxidase
UPLC-MS/MS	ultra performance liquid chromatography -tandem mass spectrometry

DANKWOORD

'It always seems impossible until it is done.' - Nelson Mandela

En zo is het maar net. Ineens is er dan het resultaat van vijf jaar onderzoek. De eindbestemming van een leerzame, uitdagende en intensieve reis, die je maar één keer in je leven maakt. Onderweg heb ik enorm genoten en ik zal nog lang en met veel plezier terugkijken. Vanzelfsprekend heb ik dit alles niet in mijn eentje gedaan. Mede dankzij de fijne samenwerking met - en interesse, betrokkenheid en steun van - alle collega's van de sectie Metabole Diagnostiek van de afdeling Medische Genetica evenals alle research collega's, heb ik mijn reis tot een succesvol einde weten te brengen. Mijn hartelijke dank hiervoor!

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CURRICULUM VITAE

Monique Albersen was born on the 27th of July in 1984 as the eldest of two daughters. She grew up in Dieren, in the east of the Netherlands and finished high school at the Stedelijk Gymnasium in Arnhem in 2002 (*cum laude*). Subsequently, Monique studied medicine at the University of Utrecht, during which she performed a six month research internship on pyridoxine dependent epilepsy and vitamin B6 transport at the Department of Metabolic and Endocrine Diseases, under supervision of Dr. Nanda M. Verhoeven-Duif and Dr. Leo Klomp. In addition, she studied body composition in children with phenylketonuria, under supervision of Dr. Gepke Visser at the Department of Pediatric Metabolic Diseases.

After graduation in 2008, Monique continued her research on vitamin B6 in a PhD program at the Section Metabolic Diagnostics of the Department of Medical Genetics, under supervision of Dr. Nanda M. Verhoeven-Duif and Prof. Dr. Nine V.V.A.M. Knoers. The results of the studies are presented in this thesis. At the same time, she successfully completed her PhD training in Clinical and Experimental Neuroscience at the Brain Center Rudolf Magnus of the UMC Utrecht. Parts of the studies presented in this thesis were conducted under supervision of Prof. Dr. Roel A. Ophoff at the University of California Los Angeles (UCLA) in Los Angeles, California (USA), for which Monique received a personal scholarship from the ESN ('Vereniging tot bevordering onderzoek Erfelijke Stofwisselingsziekten in het Nederlandse taalgebied').

Monique will continue her studies on vitamin B6 as a postdoctoral researcher after which she intends to expand her knowledge in the field of inborn errors of metabolism, while pursuing her research and discovering, admiring and enjoying everything else life has to offer!

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