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Biomedical aspects of pyridoxal 5'-phosphate availability

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1. ABSTRACT

The biologically active form of vitamin B_6, pyridoxal 5'-phosphate (PLP), is a cofactor in over 160 enzyme activities involved in a number of metabolic pathways, including neurotransmitter synthesis and degradation. In humans, PLP is recycled from food and from degraded PLP-dependent enzymes in a salvage pathway requiring the action of pyridoxal kinase, pyridoxine 5'-phosphate oxidase and phosphatases. Once pyridoxal 5'-phosphate is made, it is targeted to the dozens different apoenzymes that need it as a cofactor. The regulation of the salvage pathway and the mechanism of addition of PLP to the apoenzymes are poorly understood and represent a very challenging research field. Severe neurological disorders, such as convulsions and epileptic encephalopathy, result from a reduced availability of pyridoxal 5'-phosphate in the cell, due to inborn errors in the enzymes of the salvage pathway or other metabolisms and to interactions of drugs with PLP or pyridoxal kinase. Multifactorial neurological pathologies, such as autism, schizophrenia, Alzheimer's disease, Parkinson's disease and epilepsy have also been correlated to inadequate intracellular levels of PLP.

2. INTRODUCTION

Although the term vitamin B_6 is often employed as a synonymous of pyridoxine (PN), it actually refers to an ensemble of six substituted pyridine compounds, which also includes pyridoxal (PL), pyridoxamine (PM) and the related 5'-phosphate derivatives (Figure 1). The biologically active form of the vitamin, pyridoxal 5'-phosphate (PLP), is used as enzyme cofactor in a multitude of biochemical transformations, ranging in complexity from simple isomerizations to elaborate syntheses. Also, pyridoxamine 5'-phosphate (PMP) acts as a cofactor for the aminotransferases and in few other enzymes. Vitamin B_6-dependent (or PLP-dependent) enzymes, which are responsible for more than 160 distinct catalytic functions (about 4% of all classified enzyme activities) (1, 2), have a widespread involvement in cell metabolism, being involved in amino acids synthesis, transformation and degradation, one carbon units supply, transsulfuration, synthesis of tetrapyrrolic compounds and polyamines. Recently, PLP and pyridoxine have been shown to have a role in membrane ion transport (3-5), to bind to steroid receptors (6) and to modulate transcription factors (7). An additional function of B_6 vitamers as oxygen reactive species (ROS)

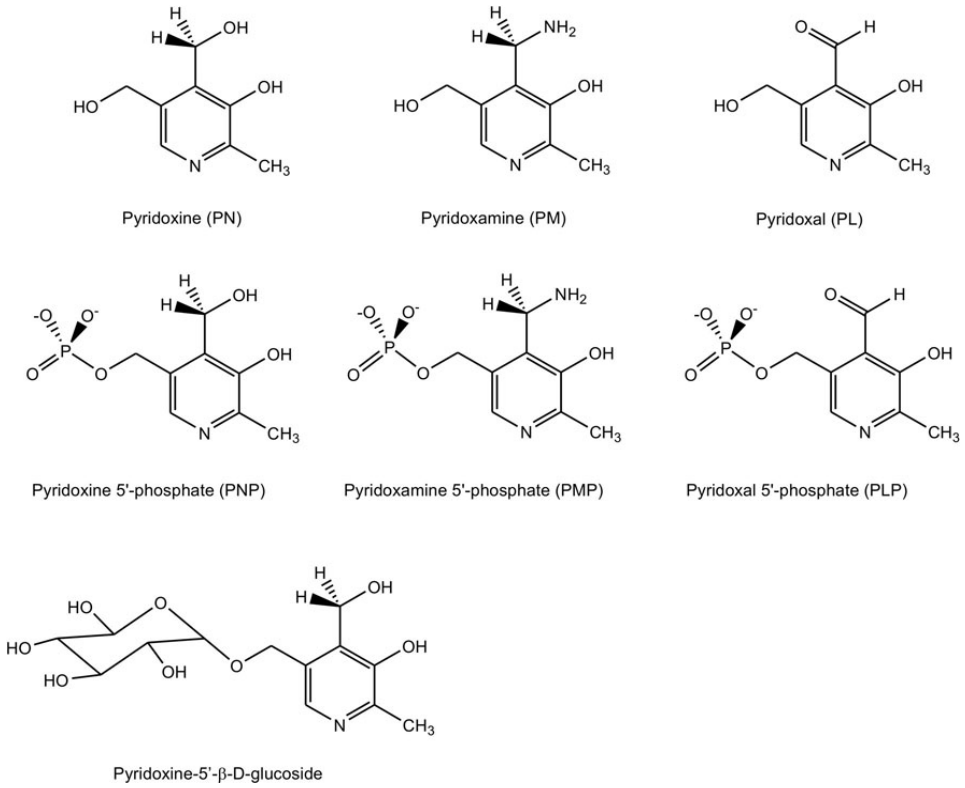

Figure 1. B_6 vitamers.

scavengers and factors able to increase resistance to biotic and abiotic stress has been demonstrated in plants (8, 9). Recently, vitamin B_6 has also been described as a new virulence factor in Helicobacter pylori, required for the chronic colonization of mice (10). Considering that mammalian hosts are unable to perform de novo vitamin B_6 biosynthesis, this finding is of particular interest for the development of new therapeutic targets against bacterial pathogens.

2.1. Role of PLP-dependent enzymes in brain function

Beside their textbook role in the synthesis and degradation of amino acids, PLP-dependent enzymes are involved in the metabolism of several neurotransmitters such as dopamine, serotonin, glycine, epinephrine, norepinephrine, D-serine, L-glutamate, gamma-aminobutyric acid and histamine, whose levels may be affected by PLP deficiency. One important example is the formation of L-glutamate and its breakdown to gamma-aminobutyric acid (GABA). L-glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the central nervous system (CNS), respectively. A significant amount of the de novo synthesized L-glutamate in the CNS is formed from alpha-ketoglutarate and branched-chain amino acids by the action of branched-chain aminotransferase (11). GABA is formed upon decarboxylation of L-glutamate catalyzed by L-glutamic acid decarboxylase (GAD), and is then consumed in the transamination reaction catalyzed by GABA aminotranferase, regenerating L-glutamate. Histamine is formed from decarboxylation of histidine, acted by histidine decarboxylase. Aromatic L-amino acid decarboxylase is involved in the formation of dopamine and serotonine. Dopamine is a precursor of epinephrine and norepinephrine. D-serine, synthesized by serine racemase from L-serine, serves as neuronal signal by activating N-methyl-D-aspartic acid (NMDA) receptors in the brain. While glycine is the primary inhibitory neurotransmitter of the spinal cord and brainstem, it has excitatory effects in the cerebral cortex owing to its agonism for the glutamatergic NMDA receptor. Glycine is formed from L-serine by serine hydroxymethyltransferase and is degraded by the glycine cleavage system, of which one of the four protein components is glycine decarboxylase, another PLP-dependent enzyme.

PLP-dependent enzymes also take part in the kynurenine oxidative pathway of tryptophan degradation, whose catabolic intermediates (kynurenic acid, 3-hydroxykynurenine and quinolinic acid) are involved in the physiological tuning of the CNS and in the etiogenesis and progression of several human neurodegenerative disorders (12, 13).

2.2. Supply of PLP to the enzymes that require it as a cofactor

Microorganisms and plants are able to synthesize PLP using two different and mutually exclusive routes and can also recycle it from protein degradation. Recycling of PLP from B_6 vitamers introduced with food is the only way all other organisms have to obtain PLP. Mammals acquire B_6 vitamers via intestinal absorption and convert them all

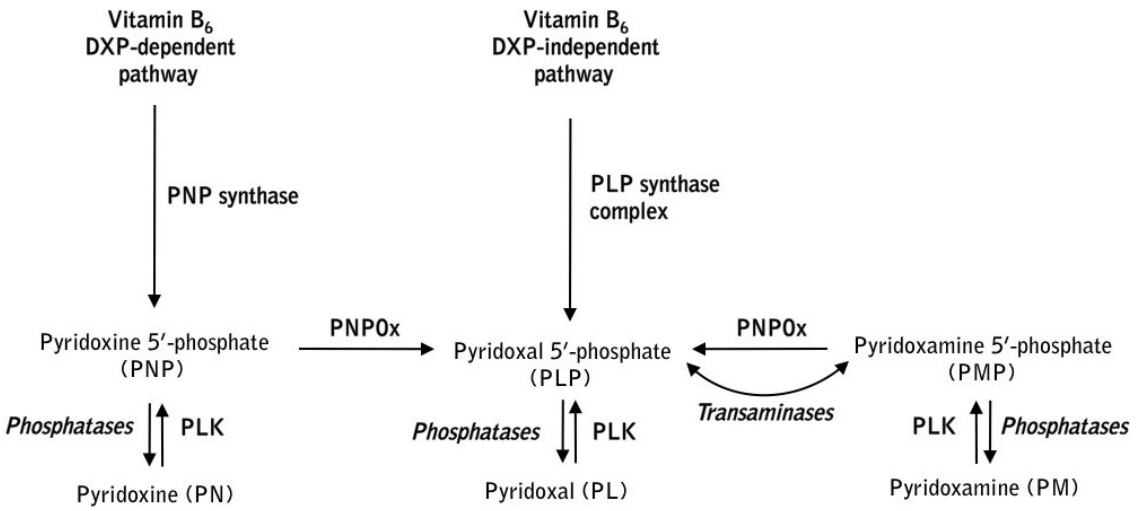

Figure 2. The vitamin B_6 salvage pathway, present in all organisms including mammals; PLK, pyridoxal kinase; PNPOx, pyridoxine (pyridoxamine) 5'-phosphate oxidase. The de novo DXP-dependent pathway is present in some eubacteria; in this pathway PNP is the first B_6 vitamer to be synthesized, starting from 4-phosphohydroxy-L-threonine and 1-deoxy-D-xylulose-5-phosphate. The de novo DXP-independent pathway is present in other eubacteria, fungi, plants and Archea; in this pathway PLP is the first vitamer to be synthesized, starting from dihydroxyacetone phosphate and ribose 5-phosphate.

into PLP through a salvage pathway (Figure 2). B_6 vitamers are absorbed from food and from the microflora, which normally colonizes the large intestine. In animal-derived foods, vitamin B_6 is mainly present as PLP, associated to glycogen phosphorylase, and in smaller amounts as PMP, while in plants it is present as PN and PN-5'-beta-D-glucoside (Figure 1) (14). PN-5'-beta-D-glucoside appears to have reduced availability because of the need for hydrolysis by an intestinal glucosidase (15). In human breast milk, vitamin B_6 is present as PLP and PL (16). The form of vitamin B_6 that is usually employed to fortify foods is pyridoxine hydrochloride, since this is the most commercially convenient.

Once ingested, PLP, PNP and PMP are dephosphorylated by the ecto-enzyme tissue-specific intestinal phosphatase, prior to absorption from the upper small intestine by a carrier-mediated system (17). The portal circulation delivers PM, PN and PL to the liver, where they are phosphorylated by the ATP-dependent pyridoxal kinase (PLK) to PMP, PNP and PLP, respectively. PNP and PMP are then both oxidized to PLP by the FMN-dependent pyridoxine (pyridoxamine) 5'-phosphate oxidase (PNPOx). PLP must return to the blood stream in order to reach all tissues. Albumin-bound PLP represents about 60% of circulating vitamin B_6, with PN, PM and PL making up the remaining part (18). While dephosphorylated B_6 vitamers can enter the cells and pass the blood-brain barrier, PLP needs to be dephosphorylated again. Tissue-specific phosphatases, expressed in placenta and germ cells (19), and the tissue-nonspecific alkaline phosphatase (TNAP) are membrane-associated enzymes responsible for this step. PL is the vitamer form that is mainly acquired by neurons; once formed by TNAP at the choroid plexus, PL is transported into the cerebrospinal fluid by an active transport mechanism and crosses the neuron plasma membranes to be rephosphorylated in the cells (18). In the cytoplasm, PL, PN and PM are converted into the 5'-phosphorylated vitamers by PLK, while PNPOx converts PNP and PMP into PLP. Once made available, PLP is somehow targeted to the dozens different apo-B_6 enzymes that are being synthesized in the cell.

The expression of mRNA of all three enzymes of the salvage pathway is ubiquitous but is highly regulated at the level of transcription in a tissue-specific manner. While PLK is expressed in all tissues, although with the presence of isoforms (20), the major sites of PNPOx expression are liver, skeletal muscle and kidneys; PNPOx is also present, to a lesser extent, in the brain, in particular in the cerebral cortex (21). PNPOx activity is absent in liver and in neurally derived tumors (22, 23). It is worth of note that the pool of free PLP in vivo must be maintained at a very low level in the body, to prevent toxic buildup. In fact, PLP being a very reactive aldehyde, easily combines with amines and thiols in the cell (24). This characteristic has been related to the neurotoxic effect of excess consumption of vitamin B_6 (19-21). In eukaryotic cells, the concentration of free PLP is maintained as low as 1 microM. One mechanism involved in PLP homeostasis is the regulation of its rate of production by PLK and PNPOx. Interestingly, it has been shown that both enzymes are able to bind their reaction product, PLP, and that their activity is finely regulated by it. Inhibition of PNPOx activity by the PLP product was observed in the E. coli enzyme, with a K_i of 8 microM (25). MgATP substrate inhibition of E. coli PLK has been observed in the presence of either PNP or PLP (26). A concurring mechanism for maintaining low levels of free PLP is expected to be its dephosphorylation by phosphatases. Catalytic conversion of pyridoxal to 4-pyridoxic acid by aldehyde oxidases and NAD-dependent dehydrogenases also keeps the level of PLP low (27).

The low free PLP concentration in the cell is not believed to be sufficient to allow for spontaneous formation of holo-B_6 enzymes (28, 29) and raises the intriguing question of how PLP is actually delivered to the apoenzymes that use it as a cofactor. The importance of vitamin B_6 in several cellular processes and in the onset of different pathologies should also be looked at from this new perspective. Studies on PNPOx and PLK suggested that once PLP is formed by these enzymes, it is unlikely to be released into solution, where it could be sequestered, and that it could be rather directly channeled to apo-B_6 enzymes (29-31). We have recently observed that a fraction of human PLK purified after expression in E. coli is complexed with PLP and that the stoichiometric amount of PLP bound to the enzyme increases if MgATP is included in the purification buffers. Our published and ongoing studies showed that the active site of PLK binds ATP and PLP tightly, forming a stable ternary complex, and that this PLP is easily transferred to apo-B_6 enzymes to form the active holoenzymes (32). Crystallographic and functional studies have shown PLP to bind to PNPOx at a tight binding site that is distinct from the active site and to be readily transferred to serine hydroxymethyltransferase (30, 33, 34). The formation of specific complexes between the oxidase or kinase and a number of different B_6 enzymes (that is a requirement for the channeling mechanism) has been observed recently in our laboratory and previously by other authors (29, 31, 32).

Failing to maintain a correct tuning between PLP biosynthesis and degradation, transport to peripheral tissues and delivering to newly formed B_6 enzymes might end up in vitamin B_6-associated pathogenesis.

3. REASONS AND CONSEQUENCES OF PLP DEFICIENCY

Several are the causes of a reduced availability of PLP in the cell, which results in the incomplete transformation of the newly synthesized, inactive apoenzymes into their active, holoenzyme form. Dietary deficiency, although rare in the western world, is a cause of insufficient vitamin B_6 consumption in developing countries, especially in infants, children, women and HIV-1 seropositive patients (35). One of the main causes of vitamin B_6 deficiency in developed countries may be the use of contraceptives. In fact, it has been proposed that the vitamin B_6 status may be adversely affected by the use of oral contraceptives (36, 37). A recent large-scale population-based study, in which plasma PLP concentration was measured in samples from nearly 8000 participants in the US National Health and Nutrition Examination Survey conducted between 2003 and 2004, showed a significant lower PLP concentration in oral contraceptive users with respect to women who had never assumed exogenous estrogens (38). The same study also showed that smoking lowers plasma PLP levels. Coeliac disease is thought to lead to a reduced absorption of B_6 vitamers (39) while renal dialysis determines their loss from circulation. Alcoholism and diabetes also cause vitamin B_6 dietary deficiency (40, 41).

Apart from weakness, irritability, insomnia and difficulty with walking, an insufficient vitamin B_6 intake has been observed to cause seizures in infants (42), EEG changes in adults (43) and has been also related to hyperhomocysteinemia (44). MCF-7 cells cultured on vitamin B_6-deficient medium have lower intracellular PLP and S-adenosyl methionine levels, and the S-adenosyl methionine to S-adenosyl-L-homocysteine ratio is also lower (45). A strong correlation has been established between high levels of homocysteine in the blood due to vitamin B_6 deficiency and cardiovascular disease (46). Dietary deficiency of vitamin B_6 has been also implicated in cerebrovascular disease (47) and cancer (48). An investigation on Wistar rat has revealed that vitamin B_6 deficiency results in the elevation of liver iron concentration and in the reduction of muscle iron concentration (49). Most probably, these alterations of tissue iron concentration are the result of an impairment of heme synthesis. 5-Aminolevulinate, the universal precursor of tetrapyrrole compounds, is derived from a reaction catalyzed by a PLP-dependent enzyme, aminolevulinate synthase, i.e. the condensation of glycine and succinyl-CoA (50). The liver is one of the highest producers of heme in the body. It was therefore hypothesized that the deficiency of heme biosynthesis caused the build up of excess iron in this organ. Macrocytic and microcytic anemia have been indeed associated with a deficiency of dietary vitamin B_6 (51). Decreased plasma PL level, loss of appetite and decreased food intake were other outcomes of vitamin B_6 deficiency in Wistar rat. Another investigation on Wistar rat has demonstrated that magnesium deficiency impairs vitamin B_6 status by decreasing intracellular Mg^{2+} concentration and thus inhibiting the activity of alkaline phosphatase, a metalloenzyme required for the uptake of PLP by tissues (52).

Vitamin B_6 supplementation has been shown to be beneficial in the management of premenstrual syndrome (53), nausea and vomiting during pregnancy (54) and treatment of carpal tunnel syndrome (55). On the other hand, large doses of vitamin B_6 were shown to have detrimental effects in experimental animals and humans. Toxicity is observed usually when the intake exceeds 200 mg/day. The current recommended dietary allowance of vitamin B_6 is 2 mg/day. Signs of toxicity occur mostly in the peripheral nervous system and include changes in gait and peripheral sensation (56). Central nervous system abnormalities are less commonly observed with excessive vitamin B_6 intake, and it has been suggested that the brain is protected from large doses of vitamin B_6 (57, 58).

Apart from dietary deficiency, secondary vitamin B_6 deficiency may result from one of the following circumstances: i) the catalytic activity of the salvage pathway enzymes may be affected by inherited mutations; ii) inborn errors in enzymes involved in certain metabolisms may lead to the accumulation of intermediates that react with PLP, reducing its availability; iii) some drugs or natural compounds binding to PLK may lead to its inactivation or reacting with PLP may reduce its availability. In all these cases, the most immediate effects

of the insufficient supply of PLP are seen on the central nervous system, in the form of several different but related neurological disorders, including convulsions and epileptic encephalopathy (59, 60).

To our knowledge, none of the reported disorders of vitamin B_6 metabolism have been ascribed to the malfunctioning of the hypothesized channeling mechanism of transfer of PLP from either PLK or PNPOx to the apo-B_6 enzymes. Of course, several pathologic conditions also occur because of inborn mutations in genes encoding PLP-dependent enzymes, which result in their missed expression, reduce their affinity for PLP or impair their regulation mechanisms and catalytic properties. These occurrences will not be considered in this review.

3.1. Inherited defects in the salvage pathway enzymes

3.1.1 Pyridoxine (pyridoxamine) 5'-phosphate oxidase deficiency

This is a newly recognized, rare disorder mimicking aromatic amino acid decarboxylase deficiency, which is presented as a neonatal epileptic encephalopathy (NEE) with seizures intractable with pyridoxine, but responding to PLP (PLP-dependent seizures). The disease, whose main feature is the onset of severe seizures within hours from a mostly premature birth, has been shown to arise from autosomal recessive mutations of the gene encoding PNPOx (OMIM 6032870) and therefore is now referred to as PNP oxidase deficiency (61). Metabolites alterations in cerebrospinal fluid, plasma and urine of affected subjects clearly indicate a deficient flux through PLP-dependent pathways: in cerebrospinal fluid, raised concentrations of 3-methoxytyrosine (3-O-methyl-dopa) and L-DOPA and lowered levels of homovanillic acid and 5-hydroxyindoleacetic acid; a raised urinary vanillactate; elevated threonine and glycine concentrations in plasma and cerebrospinal fluid; hypoglycemia and lactic acidosis (59). To date, 14 cases of patients from 8 related individuals with mutations in the PNPOx encoding gene have been reported. Surviving children are usually mentally retarded and show an abnormal dependence on vitamin B_6 in the form of PLP. At least seven mutations in the gene of PNPOx are known to result in NEE. These include homozygous missense (R95C, R95H, R229W), stop codon (X262Q), nonsense (p.A174X), splice site (IVS3-1g>a) and frameshift (c.del246T) mutations (61-66).

3.1.2. Hypophosphatasia

The indispensable role played by the tissue-nonspecific phosphatase in the cellular uptake of B_6 vitamers is evident in the onset of hypophosphatasia, a rare genetic disease whose severe forms are characterized by increased plasma concentration of PLP (67, 68) and reduced plasma concentration of pyridoxal (69). The first occurrence of hypophosphatasia was reported by Rathbun (70), who described the case of a child who died with epilepsy and rickets, with very low levels of alkaline phosphatase activity in blood and tissues. The disease results from mutations in the gene encoding the tissue-non-specific isoenzyme of alkaline phosphatase (ALPL; OMIM 171760). Most described mutations are missense mutations (see http://www. sesep.uvsq.fr/Database.html) that result in variable clinical manifestations. Six clinical subgroups may be distinguished on the basis of the age at diagnosis: perinatal lethal, prenatal benign, infantile, childhood and adult onset forms, and odontohypophosphatasia. Clinical variability has been reported in all subgroups; therefore hypophosphatasia may be regarded as a spectrum disorder (71, 72).

Tissue-nonspecific alkaline phosphatase, which is abundant in osteoblasts e chondrocytes, catalyzes the hydrolysis of inorganic pyrophosphates, which retard the growth of nascent hydroxyapatite crystals (73, 74). The intracellular accumulation of inorganic pyrophosphates, resulting from the lack of TNAP activity, accounts for the impaired skeletal mineralization observed in the subjects affected by hypophosphatasia. On top of this, affected subjects show epileptic encephalopathy and abnormal neurotransmitter metabolism (75). As mentioned above, the PL that reaches the neural cells is obtained from dephosphorylation of PLP by TNAP at the choroid plexus. TNAP deactivation therefore results in a shortage of PL supply to the neural cells and the consequent missed activation of PLP-dependent enzymes. In fact, increased levels of vanillactate are observed in hypophosphatasia patients (75), indicating a functional deficiency of aromatic amino acids decarboxylase, required for dopamine and serotonine production. Reduced GABA levels in the brain were showed in TNAP knockout mice (76), which shortly after birth developed seizures that could be prevented by the administration of pyridoxal. With human infants, good responses to treatment have been recorded also with pyridoxine (68, 69).

3.2. Inherited defects in enzymes involved in other metabolisms

3.2.1 Pyridoxine-dependent epilepsy

This is an autosomal recessive disease of probably underestimated incidence, first described in 1954 (77), that is usually presented with neonatal epileptic encephalopathy. Affected subjects depend on daily pharmacological doses of pyridoxine and if untreated die from status epilepticus. Later onsets of the disease and atypical responses to pyridoxine have been observed (59). The accumulation of pipecolic acid (Figure 3), an intermediate of lysine degradation pathway, in plasma and cerebrospinal fluid is a diagnostic marker of pyridoxine-dependent epilepsy (78, 79). The metabolic defect has been shown to originate from mutations of alpha-aminoadipic aldehyde dehydrogenase (or antiquitin, encoded by the ALDH7A1 gene), which catalyzes the oxidation of apha-aminoadipic semialdehyde, deriving from pipecolic acid, to alpha-aminoadipic acid (80, 81). The secondary PLP deficiency induced by the disease derives from the accumulation of alpha-aminoadipic aldehyde, which upon formation of an intramolecular Schiff base (L- delta^1-piperideine-6-carboxylic acid) reacts with PLP in a Knoevenagel condensation (Figure 3).

A few cases of neonatal epileptic encephalopathy have been reported to respond to treatment with folinic acid (5-formyl tetrahydrofolate, also known as leucovorin) (82, 83). These cases have been attributed to a distinct disorder

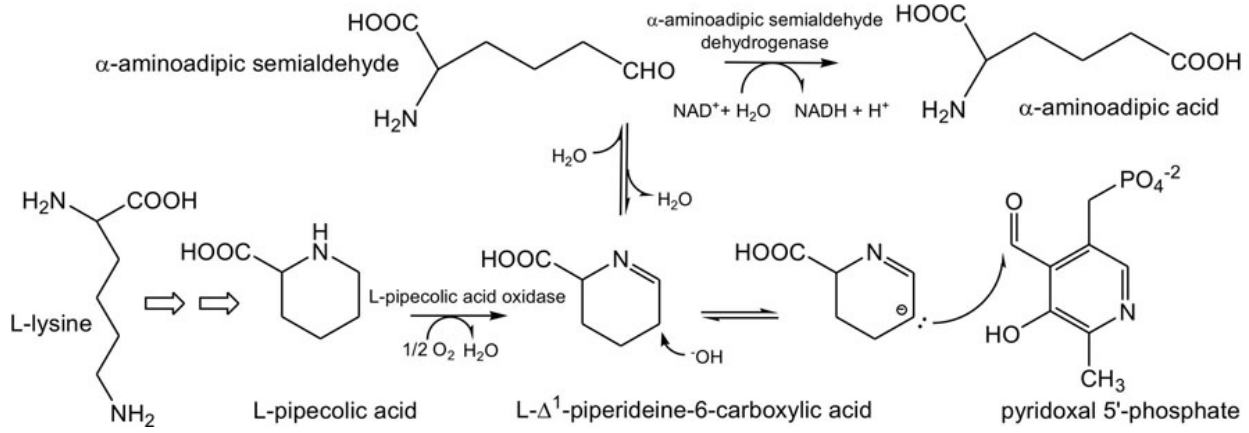

Figure 3. Lysine degradation intermediates and their interaction with pyridoxal 5'-phosphate.

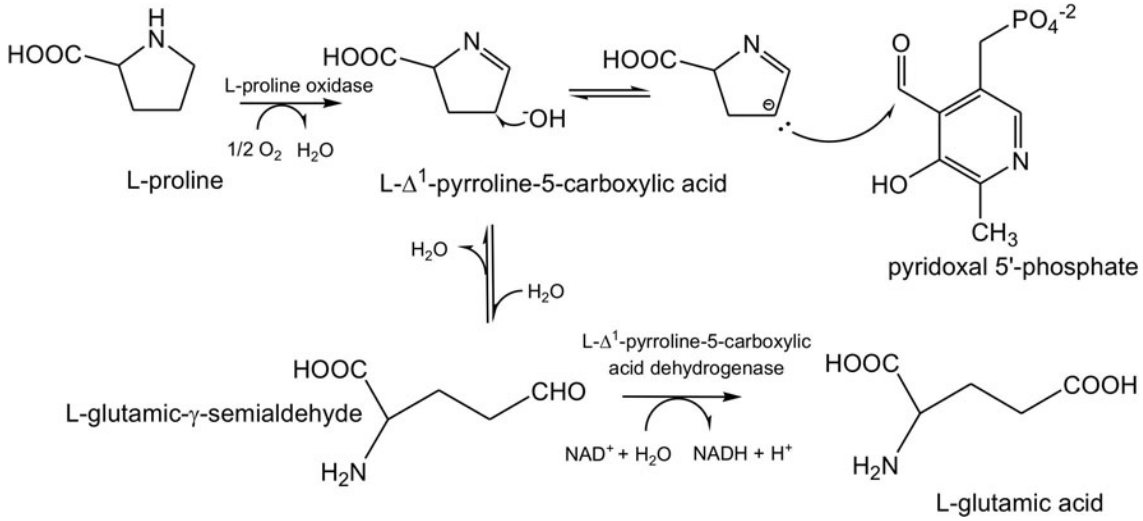

Figure 4. Proline degradation intermediates and their interaction with pyridoxal 5'-phosphate.

with respect to pyridine-dependent epilepsy, until it was demonstrated that folinic acid-responsive patients also respond to pyridoxine, are antiquitin deficient and have mutations in the ALDH7A1 gene (84). The response to folinic acid, which has been serendipitously discovered, is variable and is accompanied by a variable degree of responsiveness to pyridoxine. In our opinion, this observation, together with the fact that all folinic acid-responsive subjects showed the presence of two unidentified compounds in the cerebrospinal fluid, suggest that folinic acid-responsive seizures, although related to pyridoxine-dependent epilepsy, might not be considered the same condition.

3.2.2 Hyperprolinemia type II. This rare disorder, usually presented in childhood with fits and encephalopathy, is caused by the deficiency of the enzyme δλτα^1-pyrroline-5-carboxylate dehydrogenase, involved in proline degradation and encoded by the ALDH4A1 gene. This deficiency results in the accumulation of proline and L-δλτα^1-pyrroline-5-carboxylic acid in plasma and the consequent excessive excretion of the latter compound in urine (85). L-δλτα^1-Pyrroline-5-carboxylic acid has been shown to react in a Knoevenagel condensation of the activated C4 carbon of the pyrroline ring with the aldehyde carbon of PLP (Figure 4) (86, 87). Seizures, which manifest in approximately half of the affected individuals, respond well to pyridoxine treatment.

3.3. Drugs and natural compounds which affect PLP availability

Interference of several drugs and natural compounds with vitamin B_6 metabolism have been largely described in patients and animal studies ((88) and references therein). The effect of this interaction, which modifies the vitamin status and function, might explain some of the neurological side effects of these drugs. On the other hand, a protective effect of vitamin B_6 administration against drug toxicity has also been described (89-92).

Two mechanisms could account for B_6 hypovitaminosis induced by drugs. The first is a direct effect of compounds containing an amine or hydrazine function, which are able to react with PL or PLP. Examples

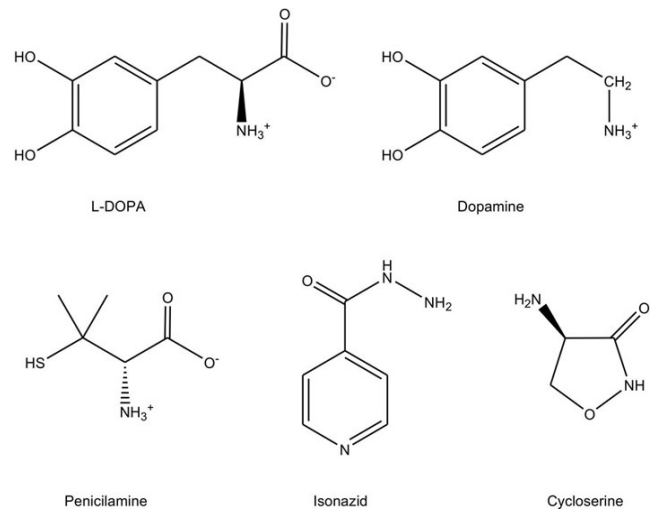

Figure 5. Compounds that interact with vitamin B_6.

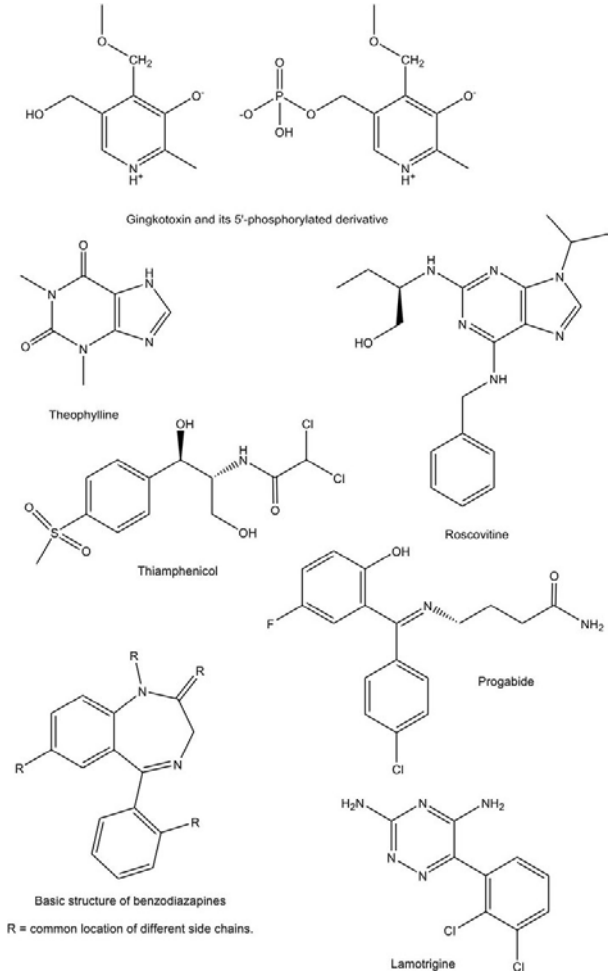

Figure 6. Compounds that interact with PLK.

are penicillamine, cycloserine, dopamine and levodopa (Figure 5). The second mechanism is the inhibition of the B_6 salvage enzymes, especially PLK, by drugs and natural compounds, such as methylxanthines, ginkgotoxin, benzodiazepines (Figure 6). Neurologic symptoms noted in severe vitamin B_6 deficiency caused by any of these

mechanisms include irritability, headache, convulsions, depression, confusion and neuropathy.

3.3.1. Drugs and natural compounds that interact with vitamin B_6

Neurotransmitters dopamine, norepinephrine, and epinephrine (collectively known as catecholamines). Aside from its natural and essential biological role, L-DOPA is also used in the clinical treatment of Parkinson's disease and dopamine-responsive dystonia. Levodopa is known to induce vitamin B_6 deficiency and to have dyskinetic side effects when large doses of pyridoxine are simultaneously administered, abolishing its therapeutic effects. The formation of a Schiff base between the amino function of L-DOPA and the aldehyde group of PLP is largely responsible of these significant side effects

Levodopa (L-DOPA). This compound is the precursor of the.

Dopamine. Contrary to its precursor L-DOPA, dopamine cannot cross the blood-brain barrier and no neurological vitamin B_6 deficiency has been linked to dopamine therapy in humans. Nevertheless, in animals intoxicated by dopamine, a rapid depletion of PLP due to the formation of a Schiff base complex between the two compounds was shown (91). This complex might also be responsible for PLK inhibition by binding to the enzyme (93).

Penicillamine. It is a metabolite of penicillin that is deprived of antibiotic properties and shows B_6 antagonistic effects. It has been shown to increase xanthurenic acid and kynurenine excretion after tryptophan load, which indicates a B_6 deficiency condition (94). This results from the reaction between the amino and sulphydryl groups of penicillamine with PLP to form of a stable thiazolidine derivative, which presents a very low affinity for the active site of PLP-dependent enzymes.

Isoniazid. This is a potent antituberculosis drug, which also has antidepressant effects. It reacts with PLP causing a vitamin B_6 deficiency (95). The pyridoxal phosphate hydrazide complex can not act as a cofactor (95) and may also inhibit pyridoxal kinase activity (96). During isoniazid treatment of tuberculosis, symptomatic pyridoxine deficiency tends to occur only in patients who are slow isoniazid inactivators or who have renal insufficiency. The most common effect is peripheral neuropathy; this and other side effects can be prevented by giving pyridoxine in a daily 50-100 mg dose.

Cycloserine. It is a highly neurotoxic compound used as antituberculosis drug in combination with isoniazid. Cycloserine-dependent convulsions can be prevented and treated with pyridoxine (92). Cycloserine reacts with PLP forming PMP, mono- and di-PLP derivatives of beta-aminoxyalanine and other covalent complexes that might also inhibit PLK.

3.3.2. Drugs and natural compounds that interact with PLK

Ginkgotoxin. Products prepared from Ginkgo biloba, a living fossil plant belonging to the Ginkgoaceae family, are top-selling phytopharmaceuticals and major botanical dietary supplements. In European medicine, G. biloba medications are used to treat neuronal disorders, and to improve brain metabolism and peripheral blood flow (97). The best known compounds derived from G. biloba are flavonoids and terpene lactones, but they also include allergenic and toxic compounds such as ginkgotoxin. Consequently, there are reports attributing beneficial as well as adverse effects to G. biloba products. Ginkgotoxin (4′-O-methylpyridoxine) is structurally related to vitamin B_6 and occurs in the seed albumen and leaves of Ginkgo biloba. Ginkgotoxin triggers symptoms called gin-nan sitotoxism: epileptic convulsions, leg paralysis, loss of consciousness and other neuronal symptoms (98). There are even reports of death due to overconsumption of Ginkgo seeds. Ingestion of raw seeds is most dangerous, while when seeds are canned or boiled the toxin content drops to only 1% compared to raw seeds. Intoxications by Ginkgotoxin can be counteracted by vitamin B_6 supplementation (97). There seems to be a connection between the main symptoms of gin-nan sitotoxism and the dysregulation of gamma-aminobutyric acid metabolism. This dysregulation has been tentatively explained by reduced GAD activity (99), however, 5'-O-phosphate ginkgotoxin has been shown to inhibit human GAD65 only at unphysiologically high concentration, while human GAD67 was not inhibited (100). Actually, the main target of ginkgotoxin seems to be PLK, which uses it as an alternative substrate, with an extremely low K_M (101). This reflects the antivitamin character of the compound and explains the in vivo depletion of PLP cofactor in the presence of ginkgotoxin. It also becomes evident why the inhibitory effect of ginkgotoxin on PLP formation by PLK can be alleviated by vitamin B_6 (namely PL) supplementation. On the other hand, it has been demonstrated that ginkgotoxin and its 5'-phosphorylated analogue do not inhibit PNPOx or pyridoxal phosphatase (102) (Figure 6).

Theophylline. Also known as dimethylxanthine, theophylline is a methylxanthine used in therapy for respiratory diseases such as chronic obstructive lung disease and asthma. Because of its numerous and poorly understood neurotoxic and pharmacological side effects, the drug is now rarely administered for clinical use. It bears structural and pharmacological similarity to caffeine. It is naturally found in tea and cocoa beans, although in amounts that are significantly lower than therapeutic doses. In a study conducted on healthy patients, theophylline administration resulted in a rapid and significant decline in both plasma and erythrocyte PLP levels, while PLK levels increased significantly. Mean erythrocyte aspartate and alanine aminotransferase activity declined drastically (50% to 70%) indicating that decreased availability of PLP can have widespread metabolic consequences. Although plasma PL levels remained normal, the threefold increase in total erythrocyte PLK activity levels did not normalize PLP levels. The effect of theophylline on vitamin B_6 metabolism

is not transitory and cannot be overcome by elevated intracellular levels of PLK. However, pyridoxine supplementation (10 mg/d for 1 week) normalized indices of vitamin B_6 status and reversed the downward trend in both aspartate and alanine aminotransferase activity levels (103). The depressed vitamin B_6 status did not seem to be responsible for the higher erythrocyte PLK activities during theophylline therapy, but rather the drug is directly responsible for elevated enzyme levels through regulation of mRNA translation and de novo synthesis of enzyme (104). Theophylline is a potent inhibitor of PLK competing with PL for enzyme binding (K_i = 8.7 microM; therapeutic theophylline concentration 55-110 microM) (105, 106). This inhibition seems to be the only mechanism responsible for unbalanced vitamin B_6 metabolism commonly found in asthmatic patients during theophylline therapy.

Roscovitine. It is a 2,6,9-substituted purine analogue that behaves as a rather selective inhibitor of several cycline-dependent kinases (CDKs), altering the growth phase or state within the cell cycle of treated cells. As a side effects, roscovitine also interacts with the non-protein kinase PLK, showing competitive binding versus ATP and PL, but not versus PLP (107). Inhibition constants are in the low micromolar range. (R)-and (S)- enantiomers of roscovitine bind to PLKs from various organisms with different specificity. Unexpectedly, the crystal structure of sheep brain PLK in complex with roscovitine found the inhibitor at the PL binding site, rather than at the expected ATP site. The comparison of PLK and CDKs binding sites for roscovitine shows a somewhat different binding environment, allowing the design of new CDK inhibitors that do not bind to PLK (108). If red blood cells were incubated with roscovitine at a final concentration of 100 microM, both enantiomers induced a significant decrease in the level of PLP in erythrocytes, whereas overall PL level remained constant. The effect of roscovitine was dose-dependent. Beside its action on vitamin B_6 balance, it seems unlike that the interaction between roscovitine and PLK could contribute to the anti-proliferative and pro-apoptotic effects of the given drug (107).

Thiamphenicol. It is the methyl-sulfonyl analogue of chloramphenicol, but is 2.5 to 5 times as potent. It is used in many countries as a veterinary antibiotic, but is available in China and Italy for use in humans. Long-term therapy is believed to cause optic neuritis and sensitive peripheral neuropathy. Besides acting as a weak inhibitor of PLK, thiamphenicol glycinate can react with the aldehyde group of PLP forming a mildly stable intermediate (88).

Benzodiazepines. These are psychoactive drugs that enhance the effect of the neurotransmitter GABA, and are used in treating anxiety, insomnia, agitation, seizures, muscle spasms and alcohol withdrawal. In general, benzodiazepines are safe and effective in the short term, although cognitive impairments and paradoxical effects occasionally occur. Interestingly, mammalian PLK has been first purified on a benzodiazepine-affinity chromatography (109) and at least some benzodiazepine-receptor ligands have been shown to bind to PLK, with inhibition features. For example, 1012-S and ethyl-beta-carboline-3-carboxylate are potent inhibitors of human PLK (IC_{50} = 2 and 5 microM, respectively), whereas other benzodiazepine-receptor ligands are much less effective (e.g. flunitrazepam and PK-11195) (110).

Antiepileptic drugs. Patients treated with antiepileptic and anticonvulsant drugs, such as progabide, carbamazepine, lamotrigine, phenytoin, phenobarbital and primidone, tend to have high plasma levels of homocysteine and low levels of folates and vitamin B_6 (111). At least some of these drugs seem to inhibit PLK activity, with a K_i in the low micromolar range. Other drugs, such as vigabatrin, reduce localized PNPOx expression in the hippocampus of seizure prone gerbils. This is a distinct effect with respect to inhibition of GABA aminotransferase. In this case, PLK is not affected by the drug (112).

4. MULTIFACTORIAL DISEASES RELATED TO PLP AVAILABILITY

In multifactorial diseases, many risk factors operating at different levels have to be taken into consideration, including biological levels (both genetic and metabolic) and societal and individual behavior. As an example, multifactorial neurological pathologies such as autism, schizophrenia, epilepsy, Alzheimer's and Parkinson's disease, have been correlated to inadequate intracellular concentration of PLP. This correlation will be taken into consideration in the next paragraph.

4.1. Autism

Several studies have been carried out on the effect of high-dose supplementation of vitamin B_6 on children and adults with autism, reporting positive benefits (113). Autistic children show abnormally high plasma levels of total vitamin B_6 (including both phosphorylated and unphosphorylated forms) compared to controls (medians of 56 versus 32 ng/ml). On the other end, levels of PLP are much lower than in control subjects. It has been found that in autistic children PLK has an increased value of K_M for pyridoxine. Thus, it appears that the reduced conversion of pyridoxal to its phosphorylated analogue results in the low levels of PLP (113). Moreover, low plasma levels of methionine, cysteine and glutathione were found in children with prototypic autistic disorder.

Normalization of PLP levels would be expected to improve mental and physical functions, and may explain many reports of improvement in autistic patients upon treatment with high-dose vitamin B_6. It could be questioned whether similar improvements would occur by simply giving PLP. However, the phosphate group being removed during digestion, PLP would likely have no additional benefits over pyridoxal. A compared study treatment with PLP or PN in autistic children had found adverse effects (worsening of behaviors) in 10% of the children receiving PLP versus none in those receiving PN. Therefore, it appears that vitamin B_6 should be given as pyridoxal HCl or pyridoxine HCl, not as PLP (113).

4.2. Schizophrenia

High serum homocysteine levels have been reported in schizophrenic patients with low folate levels (114), supporting the hypothesis that a subtle genetic defect in homocysteine metabolism may play an etiologic role in schizophrenia. A meta-analysis of eight studies has provided evidence for an association of elevated homocysteine serum levels with schizophrenia. Moreover, the elevated risk of schizophrenia has been also associated with the homozygous genotype of the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism, providing support for causality between a disturbed homocysteine metabolism and risk of schizophrenia (115). In fact, strategies that reduce homocysteine levels, such as oral administration of folic acid, vitamin B_{12} and pyridoxine, may alleviate the symptoms experienced by chronic schizophrenic patients with hyperhomocysteinemia (116). Considering that PLP is required as a cofactor for enzymes involved in homocysteine metabolism, it has been hypothesized that polymorphisms of the PNPOx encoding gene may contribute to overall genetic risk for schizophrenia. In a Japanese population study, eight single nucleotide polymorphisms (SNPs) were examined in PNPOx gene and its 5'-flanking regions in 359 schizophrenia patients and 582 control subjects. Four marker regions of PNPOx showed significant levels of allelic associations with schizophrenia (the highest was rs2325751, P=0.004). In addition, the haplotype case-control study revealed a significant association (permutation P<0.00001) between PNPOx polymorphisms and schizophrenia (117).

In another study, schizophrenic patients had lower plasma folate concentrations and elevated red blood cells levels compared to controls. Vitamin B_6, vitamin B_{12} and homocysteine levels did not differ from control. A significant dose-response relation between plasma folate concentration and risk for schizophrenia suggested a protective effect by high plasma folate concentrations. At least in this case, homocysteine levels and MTHFR 677C-T mutation did not seem to be associated with an increased risk of schizophrenia (118).

4.3. Epilepsy

Besides the obvious finding that vitamin B_6 deficiency reduces GABA concentration (promoting the onset of seizures), it has been also reported that intracerebroventricular injection of PLP causes epileptic episodes (119). Therefore, the role of PLP in the prevention, as well as in the production of convulsive seizures has still to be defined. In a Mongolian gerbil model, it was shown that PNPOx and PLK immunoreactivity was stronger in a preseizure group of seizure-sensitive animals, compared to a seizure-resistant group (120, 121). The density of both enzymes' immunoreactivity would significantly decrease 30 min after a seizure event and go back to normal in the next 12 hr. The overexpression of PNPOx and PLK may then be responsible of an excessive PLP concentration, which may enhance seizure susceptibility by diminishing the GABA levels through several mechanisms, e.g. the modification of GABAA receptors (leading to a degeneration of GABAergic neurotransmission (122)), or the enhancement of GABA uptake by neurons (123). Therefore, the excitability of the neurons in the hippocampus of seizure-sensitive animals may be unusually elevated and the salvage pathway enzymes may play an important role in this modulation. In addition, the change in PNPOx and PLK immunoreactivity following a seizure event may represent a compensatory response for reducing epileptic activity, at least in gerbils. In the same animal model, PNPOx expression and specific activity were found to be reduced in the hippocampus upon vigabatrin treatment. Vigabatrin then, besides increasing GABA concentration through its action as GABA aminotransferase inhibitor, may indirectly downregulate the expression of PNPOx in neurons, protecting the system from seizure onset. In contrast, in this case the expression of PLK was unaltered by vigabatrin treatment (112).

4.4. Alzheimer's disease, cognition, dementia, ageing

Alzheimer's disease, a neurodegenerative disorder with memory loss and progressive decline on cognitive function is the most common form of dementia. Half of the population over 85 suffers from the disease. Vascular dementia is the second most frequent type of dementia, with stroke as its major cause. Genetic and non-genetic factors, such trauma, education, stress, and nutrition, all play a role in the development of dementia. Nutrition is indeed a modifiable lifestyle factor, which may be relevant to the onset of dementia conditions. Low vitamin B_6 and elevated plasma homocysteine status is prevalent in patients with Alzheimer's disease, although it is not known if elevated plasma homocysteine or low vitamin B_6 status directly influences Alzheimer's pathogenesis or progression. It has been reported that patients with Alzheimer's disease are more likely than controls to have low plasma PLP concentrations (124).

Pharmacological doses of vitamin B_6 have been used in the hope of improving speech and language functions in children with learning difficulties. An inverse relationship between plasma pyridoxal concentration and age has been documented in several studies. Poor vitamin B_6 status and low dietary vitamin B_6 intakes and other necessary nutrients have been observed in older people, with approximately 20% of aged people having inadequate vitamin B_6 status (44). The prevailing hypothesis for the possible downward trend of vitamin B_6 status in later life involves low intake, less efficient retention, and increased catabolism of the vitamin. Also, a correlation between blood levels of B vitamins and cognitive function has been documented, and high vitamin B_6 concentration has been correlated with better performance in memorization tests (125).

There is some evidence that 20 mg of vitamin B_6 daily has an impact on vitamin B_6 levels as measured biochemically in healthy older men. Although at present there is no evidence to support the use of vitamin B_6 supplements for improving cognitive function or mood of older people, supplementation is known to improve biochemical indices of vitamin B_6 status in older men, suggesting that some may be deficient in the vitamin (126).

On the other hand, vitamin B_6 was inferred to be positively related to multiple cognitive domains and evidence was found that B_6 supplementation may improve cognitive performance in elderly men (127). It has been hypothesized that folate and vitamins B_6 and B_{12} are related to cognitive performance because homocysteine metabolism requires these vitamins, and disruption of methylation pathways may lead to cognitive impairment via the accumulation of *S*-adenosyl homocysteine, a strong inhibitor of the majority of methyltransferases. Clearly, vitamin B_6 emerged as a good predictor of cognitive performance across cognitive domains, but whether B_6 supplementation can improve cognitive performance is still to be demonstrated through ongoing longitudinal clinical trials (128).

4.5. Parkinson's disease (PD)

This is a degenerative disorder of the central nervous system that impairs motor skills, cognitive processes, and other functions. Dopamine deficiency is normally responsible for the occurring of primary Parkinson's symptoms. Current treatments are effective at managing the early motor symptoms of the disease, through the use of levodopa (L-DOPA), dopamine agonists and monoamine oxidase inhibitors. Diet also has shown some effectiveness at mitigating symptoms. However, the majority of people suffer from idiopathic PD. In a recent study, whole-genome expression profiling of isolated substantia nigra neurons from PD patients, followed by association analysis of single-nucleotide polymorphisms in differentially regulated genes, identified four differentially expressed genes, among them PLK. Intronic and 3'-untranslated region variants of PLK gene were associated with PD risk, and the up-regulation of PLK was linked to PD patients (129). Emphasis was also put on the impact of vitamin B_6 status and metabolism on Parkinson's disease risk and therapy. Successive examination of other independent patient-control series showed no significant association between PLK variants and an increased risk of disease, suggesting careful interpretation of genetic association studies (130, 131).

4.6. Malaria

It was shown many years ago that African-American people have a high frequency of low-activity PLK in red cells, compared to individuals with European ancestry. This genetic variant appears to be inherited as a single autosomal allele, and confers decreased in vivo stability on the red-cell PLK. Moreover, this racial difference was found to be tissue-specific, with leukocyte and skin fibroblast PLK activities being the same in both ethnic groups (132). Two antimalarial drugs used in the study, chloroquine and pyrimethamine, did not seem to increase PLK activity. It was suggested that the selective pressure of malaria was the cause of the lowered erythrocyte enzyme activities (133). Diet also was shown to influence PLK activity, although this alone is not enough to explain the difference seen between African-Americans and Caucasians.

A number of polymorphisms were discovered by sequencing PLK genes; among these an insertion event in the promoter region which had a significantly lower frequency in African-Americans than in either Caucasian or Asian subjects. The insert introduced a putative core promoter binding protein (CPBP) binding site adjacent to a binding site for an erythrocyte specific transcription factor. The presence of the insert was found to correlate with increased erythrocyte PLK enzyme activity, both in vivo and in vitro, and could account for the observed ethnic variation in erythrocyte PLK activity (134).

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Abbreviations: CDK: cycline-dependent kinase, CNS: central nervous system, GABA: gamma-aminobutyric acid, MTHFR: 5,10-methylenetetrahydrofolate reductase, NEE: neonatal epileptic encephalopathy, PL: pyridoxal, PLK: pyridoxal kinase, PLP: pyridoxal 5'-phosphate, PN: pyridoxine, PNP: pyridoxine 5'-phosphate, PNPOx: pyridoxine (pyridoxamine) 5'-phosphate oxidase, PM: pyridoxamine, PMP: pyridoxamine 5'-phosphate, TNAP: tissue-nonspecific alkaline phosphatase.

Key Words: Vitamin B_6, Pyridoxal 5'-Phosphate, Salvage Pathway, Pyridoxal Kinase, Pyridoxine 5'-Phosphate Oxidase, Plp Deficiency, Neurological Disorders, Review

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