# Part 1: The role of vitamin B6 in one-carbon metabolism, oxidative stress, immune system regulation and carcinogenesis

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Vitamin B6 is a well known cofactor in over 100 metabolic reactions, among which one-carbon metabolism, and is shown to play a role in oxidative stress, immune system regulation and carcinogenesis. In one-carbon metabolism, vitamin B6 is a co-factor for serine hydroxylmethyltransferase (SHMT), cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). One-carbon metabolism is important in DNA synthesis, DNA repair, DNA methylation, protection against oxidative stress and detoxification. Therefore, adequate levels of vitamin B6 are required for maintenance of these processes.
Vitamin B6, especially the vitamer pyridoxamine, is shown to have anti-oxidative capacities by scavenging reactive oxygen species (ROS), reactive carbonyl species (RCS) and chelation of redox-active metal ions.
In vitamin B6 deficiency, suppression of development of lymphoid organs, lymphocyte proliferation, cytotoxicity, delayed type hypersensitivity reaction, skin transplant rejection, antibody production and production of interleukins involved in a T-helper 1 response, are shown in in vitro and animal studies. The effects of vitamin B6 status on one-carbon metabolism are the most hypothesized mechanism of the aberration of the regulation of the immune system, found in vitamin B6 deficiency.
Vitamin B6 is shown to protect against (colorectal) carcinogenesis. Several mechanisms are hypothesized, such as aberration of one-carbon metabolism, expression of genes involved in cell proliferation, detoxification of carcinogenic compounds, protection against oxidative stress and angiogenesis, oxidative stress, inflammation and nitric oxide synthesis.

## Introduction

### Vitamin B6 structure and metabolism

Vitamin B6 consists of a 2-methyl-3-hydroxypyridine and is present in the human body in six isoforms. These vitamers have different chemical structures on the C4 atom of the pyridine: pyridoxine (PN) has a hydroxymethyl group, pyridoxal (PL) an aldehyde group and pyridoxamine (PM) an aminomethyl group. These structures, shown in figure 1, can be phosphorylated by pyridoxal kinase at the hydroxymethyl group bound to the C5 atom to form three phosphate esters: pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP).[1-3]

In the human body, vitamin B6 cannot be synthesized. Therefore, the main sources are animal- and plant-derived food products, such as vegetables, whole grain cereals, nuts and muscle meats and the production by bacteria in the large intestine.[1-4] The main vitamer present in animal-derived foods is PLP, but it contains also a small amount of PMP. In plant-derived food products PN, PNP and pyridoxine glucoside are the main vitamers.

*Abbreviations*: 3-DG, 3-deoxyglycosone; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; AGEs, Advanced glycation end-products; ALEs, Advanced lipoxidation end-products; APCs, Antigen presenting cells; CBS, Cystathionine β-synthase; CGL, Cystathionine γ-lyase; cSHMT, Cytoplasmic serine hydroxylmethyltransferase; CTLs, Cytotoxic T-lymphocytes; DNP-OVA, Dinitrophenylated ovalbumin; DPD, Deoxypyridoxine; DTH, Delayed-type hypersensitivity; dTMP, Deoxythymidylate 5'-monophosphate; dUMP, Deoxyuridylate 5'-monophosphate; FAD, Flavin adenine dinucleotide; GLA, Glycolaldehyde; GO, Glyoxal; GPx, Glutathione peroxidase; GSH, Glutathione; GSSG, Glutathione disulfide; HNE, Hydroxynonenal; IFN, Interferon; Ig, Immunoglobulin; IL, Interleukin; (i)NOS, (Inducible) nitric oxide synthase; MDA, Malondialdehyde; MGO, Methylglyoxal; MHC, Major histocompatibility complex; MS, Methionine synthase; mSHMT, Mitochondrial serine hydroxylmethyltransferase; MTHFR, Methylenetetrahydrofolate reductase; NK cells, Natural killer cells; NO, Nitric oxide; PL, Pyridoxal; PLP, Pyridoxal 5'-phosphate; PM, Pyridoxamine; PMP, Pyridoxamine 5'-phosphate; PN, Pyridoxine; PNP, Pyridoxine 5'-phosphate; PNPO, Pyridox(am)ine-5'-phosphate oxidase; PPD, Purified protein derivate; RCS, Reactive carbonyl species; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, Serine hydroxylmethyltransferase; SOD, Superoxide dismutase; SPI-3, Serine protease inhibitor clade A member 3; TE cells, Thymic epithelial cells; Th cells, T helper cells; THF, Tetrahydrofolate; TNF, Tumor necrosis factor; TS, Thymidylate synthase.

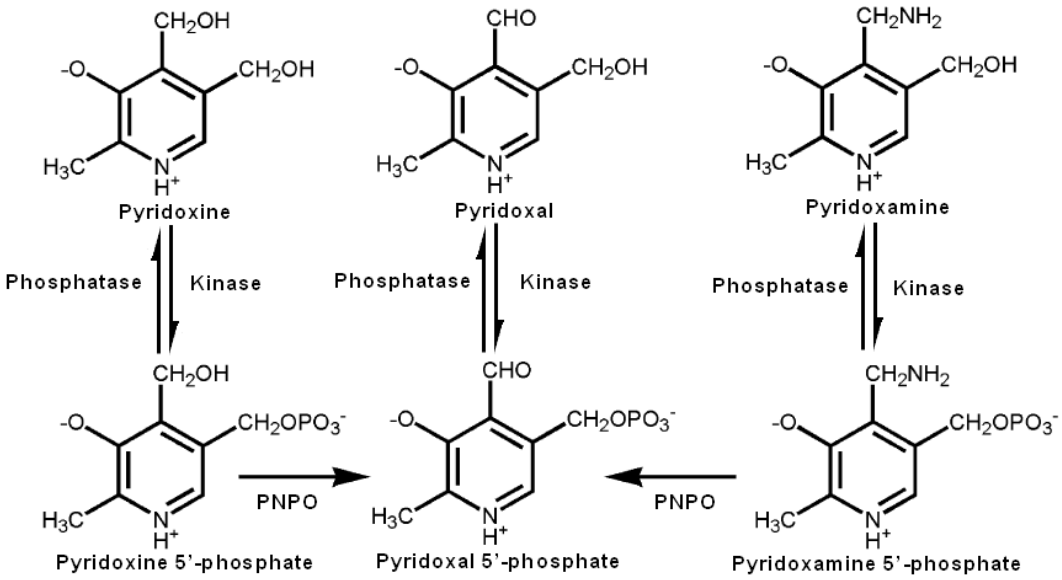


*Figure 1:* Interconversions of the six vitamers of vitamin B6. Modified from Depeint et al[3]. PNPO: pyridox(am)ine 5'-phosphate oxidase

In supplementation and studies to investigate the effect of vitamin B6, PN is the most used vitamer. Bacteria can produce PLP directly or indirectly through PNP.[1-3] The phosphorylated vitamers cannot be transported across cell membranes. Therefore, they are dephosphorylated in the intestine by phosphatases. The unphosphorylated forms are transported to the liver, where they are rephosphorylated by pyridoxal kinase. PLP is the most active vitamer and therefore in the liver, PNP and PMP are converted to PLP by pyridox(am)ine 5'-phosphate oxidase (PNPO), as shown in figure 1. PLP is transported through the blood bound to albumin. PLP is dephosphorylated again before entering cells and rephosphorylated intracellularly to regain the active cofactor. In the liver, vitamin B6 can be converted to 4-pyridoxic acid, which is excreted in urine.[2, 3] In some studies, 4-deoxypyridoxine (DPD) is used to reach a vitamin B6 deficient state. DPD can be phosphorylated by pyridoxal kinase and competes with PLP by binding to the PLP-binding sites of PLP-dependent enzymes. Therefore, DPD is often called a vitamin B6 antagonist.[5]

**Vitamin B6 functions**

PLP, the active form of vitamin B6, is an essential coenzyme in 100-150 metabolic reactions in not only amino acid and neurotransmitter metabolism [1, 2], but also in other systems in the human body. Examples of additional functions are functions as cofactor in carbohydrate metabolism[2], one-carbon metabolism[3], fatty acid metabolism[1], heme synthesis[1] and involvement in regulation of gene expression[6] and regulation of the immune system[5, 7]. Also anti-oxidative properties[8] and effects on carcinogenesis[9, 10] have been described. PLP functions not only as cofactor for enzymes, but also influences these systems by direct binding of its aldehyde group to amino groups of proteins, mostly the ε-amino group of lysine, forming a Schiff base.[3, 6] In vitamin B6 deficiency, the above described functions can be disturbed. Symptoms of vitamin B6 deficiency vary with its severity. In mild to moderate deficiency, symptoms are abdominal discomfort, nausea and headache, but in severe deficiency, epileptic seizures, anemia, renal failure, dermatitis and vomiting occur[3, 11].

The role of vitamin B6 in oxidative stress, the regulation of the immune system and carcinogenesis are studied and described. The role of vitamin B6 in one-carbon metabolism is thought to be important in the regulation of the immune system and carcinogenesis and possibly also in oxidative stress. Therefore, the role of vitamin B6 in one-carbon metabolism is described first.

**One-carbon metabolism**

One-carbon metabolism consists of a couple of reactions in amino acid metabolism in which one-carbon groups are exchanged. It is divided in two pathways: a remethylation pathway and a transsulfuration pathway, as shown in figure 2.[12, 13] In both pathways vitamin B6 is a cofactor of several enzymes. One-carbon metabolism plays a role in DNA synthesis, DNA methylation, detoxification and protection against oxidation[12, 14].

One-carbon groups are methyl (-$CH_3$), methylene (-$CH_2$-), formyl (-CHO), formimino (-CHNH) and methenyl (-CH=) groups. Amino acids involved in one-carbon metabolism are homocysteine, methionine, serine, glycine and cysteine. Serine originates from a glycolysis intermediate, 3-phosphoglycerate, and can be converted into glycine, a reaction catalyzed by the PLP-dependent enzyme, serine hydroxylmethyltransferase (SHMT). Serine can also

react with homocysteine to form cysteine, a pathway catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL), which are both PLP-dependent enzymes. Homocysteine is the precursor for methionine.[14]

***Remethylation pathway***

The most important carrier of one-carbon groups is tetrahydrofolate (THF), which is derived from folic acid through dihydrofolate, see figure 2. Serine donates one-carbon groups to THF and is the most important source of one-carbon groups. When serine is converted into glycine, 5,10-methylenetetrahydrofolate (5,10-MTHF) is formed from tetrahydrofolate and the methylene group of the side-chain of serine. This reaction is catalyzed by serine hydroxylmethyltransferase (SHMT), a PLP-dependent enzyme.[12-14] SHMT is located in the cytoplasma and the mitochondria, called cytoplasmic SHMT (cSHMT) and mitochondrial SHMT (mSHMT), respectively.[3, 14-16]

THF can be regenerated from 5,10-MTHF through 5-methyltetrahydrofolate (5-MTHF), a reaction catalyzed by methylenetetrahydrofolate reductase (MTHFR), a flavin adenine dinucleotide (FAD, a derivate of riboflavin)-dependent enzyme, as shown in figure 2. 5-MTHF is the circulating form of folate in the human body[12]. The methyl group of 5-MTHF is transferred to homocysteine to form methionine and simultaneously THF is regenerated. This reaction is catalyzed by methionine synthase (MS), a methylcobalamin (a derivate of vitamin B12)-dependent enzyme, see figure 2.[13, 14]

Tetrahydrofolate and its derivatives can act as acceptor or donor of one-carbon groups in metabolic reactions, but energetically, these are not the most favourable reactions. Methionine can react with the adenosyl group of ATP to form S-adenosylmethionine (SAM) and this intermediate can donate its methyl group easily. Therefore, S-adenosylmethionine is the most important methyl group donor in methylation reactions. When SAM donates its methyl group, S-adenosylhomocysteine (SAH) is formed, which can be converted back to homocysteine and adenosine.[13, 14]

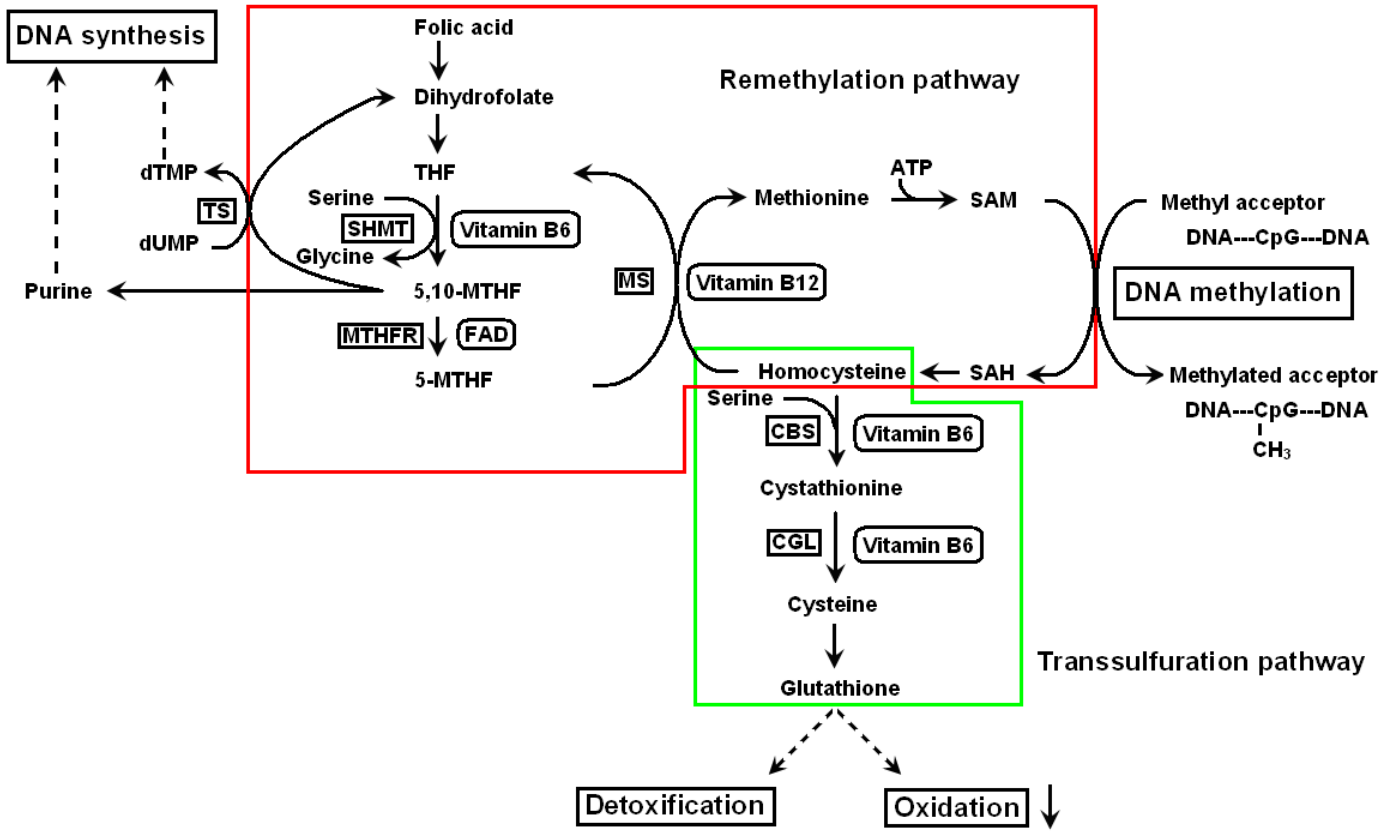


*Figure 2:* Overview of the one-carbon metabolism. The one-carbon metabolism consists of the remethylation pathway (red) and the transsulfuration pathway (green). Vitamin B6 is the cofactor of SHMT in the remethylation pathway and of CBS and CGL in the transsulfuration pathway. Modified from Larsson et al[10]. 5,10-MTHF: 5,10-methylenetetrahydrofolate, 5-MTHF: 5-methyltetrahydrofolate, ATP: adenosine triphosphate, CBS: cystathionine β-synthase, CGL: cystathionine γ-lyase, dTMP: thymidylate 5'-monophosphate, dUMP: deoxyuridylate, FAD: flavin adenine dinucleotide, MS: methionine synthase, MTHFR: methylenetetrahydrofolate reductase, SAH: S-adenosylhomocysteine, SAM: S-adenosylmethionine, SHMT: serine hydroxylmethyltransferase, THF: tetrahydrofolate, TS: thymidylate synthase.

In vitamin B6 deficiency, reduced cSHMT and mSHMT activity are shown.[16-19] Hypothetically, reduced SHMT activity could decrease 5,10-MTHF and 5-MTHF availability for DNA synthesis and remethylation of homocysteine, respectively. Studies indicate that in vitamin B6 deficiency, the methylation capacity is reduced, but that is uncertain.[15-18]

Several studies[16-19] showed a decreased activity of both isozymes of SHMT in hepatocytes of rats, fed a vitamin B6 deficient diet,[17, 19] and lymphocytes of humans (n=9) on a vitamin B6 deficient diet[18], compared to rats and humans on a normal diet. Reduced activity of SHMT was also shown in human MCF-7 cells (breast cancer cell line) cultured in vitamin B6 deficient medium compared to cells cultured in medium containing adequate levels of vitamin B6[16]. Martinez et al[17] and Perry et al[16] both showed that cSHMT activity was more affected by vitamin B6 deficiency than mSHMT activity.

Despite the fact that these studies showed a decreased SHMT activity in vitamin B6 deficiency, it is unclear whether vitamin B6 status really affects the flux through the remethylation pathway. Martinez et al[17] and Perry et al[16] showed a decreased concentration of SAM in rats with a vitamin B6 deficient diet and in cells on vitamin B6 depleted medium, respectively. Perry et al[16] found also a decreased SAM:SAH ratio, which is representative for a decreased methylation capacity. Therefore, they both concluded that vitamin B6 deficiency lowers the availability of methyl groups derived from serine for the remethylation of homocysteine.
In contrast, Davis et al[18] found that levels of metabolites involved in one-carbon metabolism were not altered in lymphocytes of humans on a vitamin B6 deficient diet compared to lymphocytes of humans on a normal diet. They concluded that the remethylation of homocysteine is not affected by moderate vitamin B6 deficiency and suggested that the normal capacity of SHMT exceeds the needed capacity.
Nijhout et al[15] made a mathematical model of one-carbon metabolism and investigated the effect of lowering the activity of SHMT, CBS and CGL, the PLP-dependent enzymes involved in one-carbon metabolism. They found that lowering the activity of SHMT had only a small effect on levels of metabolites involved in one-carbon metabolism and concluded that vitamin B6 status does not affect the remethylation of homocysteine. Thus, in literature there is an indication that flux through the remethylation pathway of one-carbon metabolism is decreased in vitamin B6 deficiency, but further investigations are required.

The remethylation pathway has multiple functions, for example in DNA synthesis, repair and methylation. Possibly, in vitamin B6 deficiency, these functions are affected, because of reduced SHMT activity and SAM production.

*DNA synthesis* – Derivatives of THF also serve as methyl donors in the synthesis of DNA bases, as shown in figure 2[14]. DNA bases are divided into purines (adenine and guanine), and pyrimidines (cytosine and uracil in RNA and thymine instead of uracil in DNA)[20]. In de novo synthesis of purines, the 2$^{nd}$ and 8$^{th}$ C-atoms are derived from formate or 10-formyl-tetrahydrofolate, which both originate from THF.[12, 14, 16]

The nucleotides of RNA and DNA, called (deoxy)ribonucleotides, consist of one of the four bases connected to ribose and deoxyribose, respectively. In the final step of deoxythymidylate (dTMP), the thymine-derived deoxyribonucleotide, synthesis, 5,10-MTHF serves as a methyl donor. Therefore, deoxyuridylate (dUMP), a uracil-derived deoxyribonucleotide, is converted to dTMP by thymidylate synthase (TS) and THF is regenerated simultaneously from 5,10-MTHF, as shown in figure 2.[14]

In vitamin B6 deficiency, SHMT activity is shown to be decreased, but its effect on the flux through the remethylation pathway is unclear. Hypothetically, the availability of 5,10-MTHF, formate and 10-FTHF for nucleotide synthesis could be reduced and thereby possibly also RNA and DNA synthesis.

Some studies have shown evidence that vitamin B6 deficiency affects synthesis of DNA and RNA. A study of Trakatellis et al[21] showed decreased incorporation of labeled C-atoms derived from serine and adenine in DNA and RNA, and labeled H-atoms derived from thymidine, in DNA of rats fed a vitamin B6 deficient diet compared to rats fed a normal diet. The reduced incorporation of labeled C-atoms derived from serine indicate reduced nucleotide synthesis from the one-carbon metabolism. But these results also suggest that DNA and RNA synthesis are reduced in vitamin B6 deficiency, because of reduced incorporation of labeled C- and H-atoms from adenine and thymidine, respectively, precursors of nucleotides which are not involved in one-carbon metabolism. It is most likely that the reduced incorporation of these precursors is caused by less DNA synthesis, but possibly vitamin B6 affects the incorporation in another way. Montjar et al[22] also showed a decreased incorporation of labeled orotic acid, a precursor of uracil, in mRNA and rRNA in hepatocytes of rats fed a vitamin B6 deficient diet compared to rats fed a normal diet. This might suggest that RNA synthesis also is reduced in vitamin B6 deficiency.

*DNA repair* – The methylation of dUMP is important for the recognition and repair of DNA mutations. Normally, cytosine can be deaminated spontaneously to uracil. Therefore, cytosine-guanine base pairs can mutate in daughter DNA strands to uracil-adenine base pairs. Thymine can be discriminated from deaminated

cytosine, because thymine is methylated. One of the DNA repair systems, carried out by the enzyme uracil DNA glycosylase, can recognize and remove uracil in DNA by making a nick in the DNA strand. Then, uracil can be replaced by cytosine. In this way, the spontaneously deaminated cytosines are recognized and distinguished from thymine. Thus, the methylation of dUMP to dTMP is important for DNA repair.[12, 14]

Vitamin B6 deficient subjects are thought to be more susceptible to DNA and chromosome breaks by the same mechanism as described in folate deficiency. Blount et al[23] found that in folate deficiency, 5,10-MTHF needed for dUMP methylation, is decreased, and the dUMP/dTMP ratio is increased. They found increased misincorporation of uracil in DNA and hypothesized the following mechanism; Because uracil DNA glycosylase makes a nick in DNA strands to replace uracil, single-strand DNA breaks are more likely. When uracil misincorporation increases, misincorporation in two complement DNA strands is more likely and even double-strand DNA breaks could occur. In this way, folate deficiency could cause DNA and chromosome breaks and instability. Mashiyama et al[24] hypothesized that this mechanism also could play a role in vitamin B6 deficiency. Hypothetically, in vitamin B6 deficiency, availability of 5,10-MTHF could be decreased because of reduced SHMT activity. Reduced availability of 5,10-MTHF could cause reduced methylation of dUMP and thereby increased misincorporation of uracil, causing DNA and chromosome instability. However, Mashiyama et al found no association between vitamin B6 status and uracil misincorporation in humans (n=12), but suggested that the vitamin B6 deficiency was not severe enough to find significant associations.[12, 14, 23, 24]

*DNA methylation* – S-adenosylmethionine (SAM) can function as methyl group donor for the methylation of DNA bases. Methylation of DNA is related to decreased gene transcription. Around gene promoters the repetitive sequence cytosine-guanine (CG), called CpG islands, is highly preserved during evolution. In these CpG islands, methylation of cytosine nucleotides to 5-methyl cytosine by DNA methyltransferases causes binding of proteins to DNA. Normally, DNA is wrapped around histones, structures involved in accessibility of DNA for transcription. Proteins which bind these methylated CpG sites, are mostly involved in deacetylation of histones, thereby lowering the accessibility of DNA for transcription. In this way, methylation of DNA bases can suppress gene transcription.[12, 14, 20, 25]
Also non-coding DNA is highly methylated, preventing transcription of these regions. Formation of potent harmful transcripts of non-coding DNA, such as transcripts of repetitive elements, inserted viral sequences and transposons, is prevented by methylation of non-coding DNA[25].

As described previously, in literature there is an indication that in vitamin B6 deficiency, SAM concentration and SAM:SAH ratio is reduced and thereby the methylation capacity.[16, 17] Hypothetically, in that way vitamin B6 deficiency could account for DNA hypomethylation, thereby increasing gene transcription.

***Transsulfuration pathway***
Serine and homocysteine together can be converted to cysteine in two enzymatic reactions. Serine and homocysteine are irreversibly condensated to cystathionine by cystathionine β-synthase (CBS), a PLP-dependent enzyme. Cysteine and α-ketobutyrate are hydrolysed from cystathionine, a reaction catalyzed by cystathionine γ-lyase (CGL), also a PLP-dependent enzyme, as is shown in figure 2.[3, 12-14, 17, 26]

Cysteine can be converted to glutathione (GSH), which is a tripeptide consisting of cysteine, glycine and glutamate. Glutathione has an important role in detoxification and protection against oxidative stress, through for example reduction of hydrogen peroxide and lipid hydroperoxides. In this reaction, GSH is converted to glutathione disulfide (GSSG, two GSH molecules with a S-S bridge), a reaction catalyzed by glutathione peroxidase (GPx), as shown in figure 3. GSSG is recycled with NADPH, derived from the pentose phosphate pathway, by GSH reductase to GSH and $NADP^+$.[12-14, 26]

The thiol group, a -SH group bound to a C-atom, of the cysteine residue of GSH, functions as a sulfhydryl (-SH) buffer. In oxidative stress thiol groups of proteins are susceptible to oxidation to disulfides (-S-S-). This is prevented by the conversion of GSH to GSSG catalysed by GPx. GSH maintains the reduced state of thiol group-containing proteins by preventing intra- and intermolecular cross-linking by disulfide bridges. In this way, GSH formed in the transsulfuration pathway protects against oxidative stress.[3, 12-14, 26]

Glutathione is also important in several other mechanisms, such as detoxification of formaldehyde and xenobiotics, foreign substances. Formaldehyde is a product from the metabolism of for example methionine, methanol or several xenobiotics. It is a carcinogenic substance and can be converted with glutathione to S-formyl-glutathione, a reaction catalyzed by formaldehyde dehydrogenase. Glutathione can also prevent toxic effects of metabolites and xenobiotics by reacting directly with them.[27]

Hypothetically, in vitamin B6 deficiency, the flux through the transsulfuration pathway could be decreased, but in literature both, stimulation and inhibition of the pathway, are described. Martinez et al[17] and Lima et al[28] both showed a decreased CGL activity in rats fed a vitamin B6 deficient diet compared

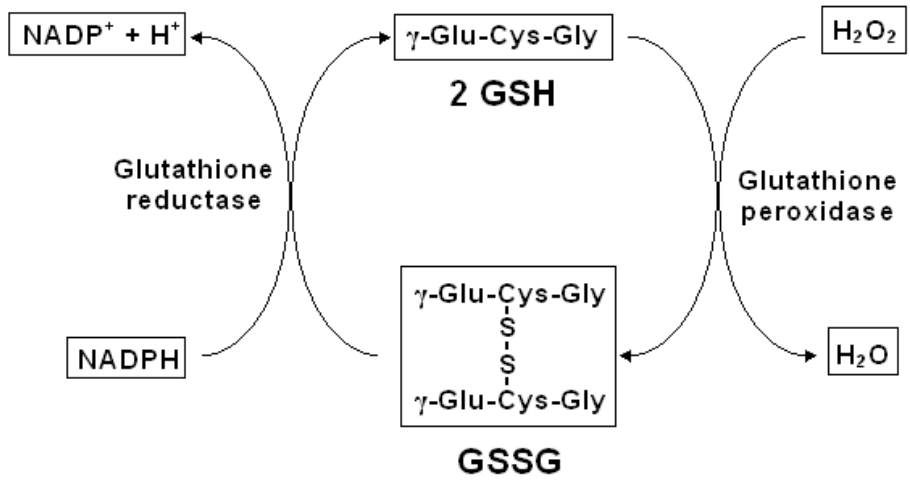


*Figure 3:* The anti-oxidative mechanism of glutathione. Two glutathione tripeptides can be converted by glutathione peroxidase to glutathione disulfide, thereby reducing hydrogen peroxide to water. Glutathione can be resynthesized from glutathione disulfide by glutathione reductase by conversion of NADPH, derived from the pentose phosphate pathway, to $NADP^+$ and $H^+$. Modified from Baynes and Dominiczak[26]. GSH: glutathione, GSSG: glutathione disulfide, NADP: nicotinamide adenine dinucleotide phosphate.

to rats fed a normal diet, but CBS activity was unaffected. Martinez et al[17] found a decreased formation of cystathionine and cysteine, indicating a decreased flux through the transsulfuration pathway in vitamin B6 deficiency. Conversely, Lima et al[28] and also the mathematical model of Nijhout et al[15] showed increased levels of cystathionine and glutathione. They suggested that vitamin B6 deficiency enhances oxidative stress and that, oxidative stress stimulates the transsulfuration pathway.[15]

In conclusion, it is clear that the transsulfuration pathway is affected by vitamin B6 status, but it is unknown whether a deficiency stimulates or inhibits the pathway. Hypothetically, a decreased CGL activity could indirectly decrease GSH formation, thereby decreasing detoxification and protection against oxidative stress.

## The role of vitamin B6 in Oxidative stress

**Oxidative stress**

Oxidative stress is an imbalance of oxidation reactions and antioxidative mechanisms in favour of oxidation (table 1). The main oxidant in the human body is oxygen. Oxygen has a high endogeneous activation energy, but can easily be activated and reduced by redox-active metal ions. Only 1% of the oxygen in the body is converted to reactive oxygen species (ROS). ROS are the highly reactive reduced forms of oxygen, as shown in figure 4, which are useful in for example inflammation reactions and as enzyme substrates, but they can also harm biomolecules. Therefore, antioxidative mechanisms prevent the formation of ROS and other products of oxidative stress and decrease oxidative damage in several other ways.[26] Vitamin B6 is thought to have antioxidant properties.

*Reactive oxygen species (ROS)*

ROS are the intermediates of oxygen reduction to water and can be formed in the human body in three ways:

- By reduction of oxygen catalysed by redox-active metal ions, such as iron and copper;
- In the oxidative phosphorylation pathway in mitochondria;
- In other enzymatic oxidation reactions.

In order of the reductive state, ROS are superoxide ($O_2^{\cdot-}$), hydroperoxyl ($HOO\cdot$), hydrogen peroxide ($H_2O_2$), hydroxyl ($OH\cdot$) and hydroxide ($OH^-$), as shown in figure 4. Superoxide is mostly reduced to hydrogen peroxide, but in 0.1% it is converted to hydroperoxyl. Reduction of hydrogen peroxide results in its cleavage into hydroxyl and hydroxide, which both can be reduced with hydrogen to water, as shown in figure 4. In the presence of lightenergy in for example the skin, oxygen can also be converted to singlet oxygen ($^1O_2$), a process called photosensitization.[26]

*Reactive nitrogen species (RNS)*

ROS can also react with nitric oxide ($NO\cdot$), a radical important in the regulation of oxidative phosphorylation and vasodilatation, to form reactive nitrogen species (RNS), which are also highly oxidative. Superoxide and nitric oxide together can be converted to the RNS peroxynitrite ($ONOO^-$), which possibly can be cleaved into $OH\cdot$ and $NO\cdot_2$. The latter product, also a RNS, is formed by the reaction of $NO\cdot$ with $H_2O_2$ catalysed by peroxidase or myeloperoxidase.[26]

*Oxidation of biomolecules*

In oxidative stress, biomolecules, such as lipids, carbohydrates, proteins and nucleic acids, can be non-enzymatically oxidized by ROS and RNS and thereby their structure and functioning can be disturbed.[26]

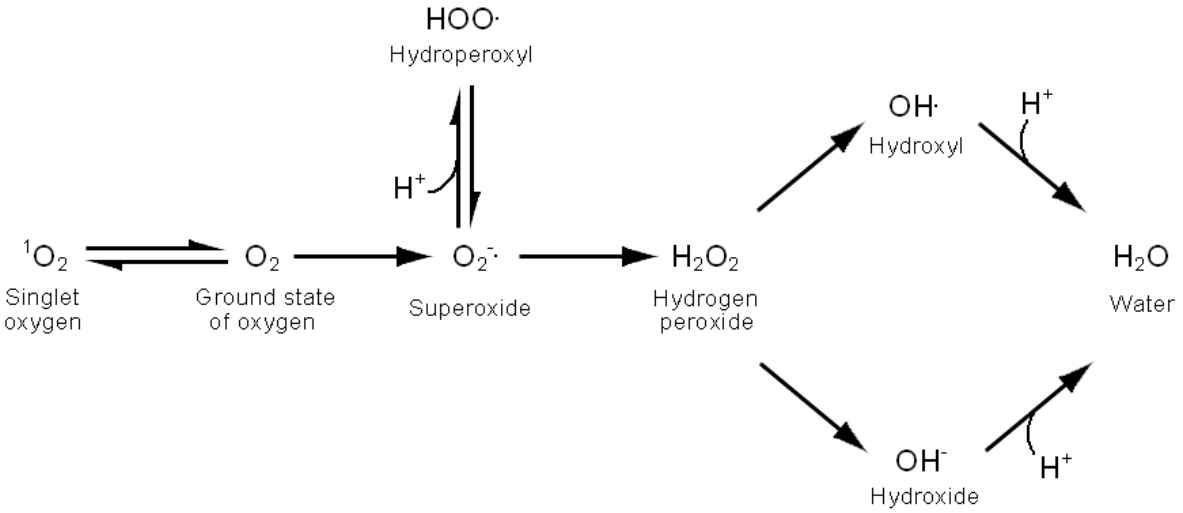


*Figure 4:* Oxygen can be reduced through reactive oxygen species to water. Modified from Baynes and Dominiczak[26].

Lipids can be oxidized by ROS and RNS in a chain of lipid peroxidation reactions. Polyunsaturated fatty acids, phospholipids and cholesterol esters in cell membranes are highly susceptible to oxidation. The main products of lipid peroxidation are reactive carbonyl species (RCS), malondialdehyde (MDA) and hydroxynonenal (HNE) and these molecules can react with proteins to form advanced lipoxidation end-products (ALEs), as shown in figure 5. During lipid peroxidation other ROS are formed, such as peroxyl and alkoxyl radicals.[8, 26]

Autoxidation of carbohydrates also results in carbonyl compounds, which can react with proteins to advanced glycation end products (AGEs), as shown in figure 5.[26] Carbohydrates can also directly react with proteins in a glycation reaction. The aldehyde-groups of carbohydrates and the ε-amino-groups of amino acids, such as lysine, or the N-terminal α-amino groups of proteins, can be reversible condensated to a Schiff base, which irreversibly can be converted to an intermediate, called the Amadori intermediate, as shown in figure 5. In several oxidation, dehydration and condensation reactions, this intermediate is converted to AGEs. During autoxidation of carbohydrates and oxidation of the Amadori intermediate the major ROS formed are superoxide, hydrogen peroxide and the hydroxyl radical. The pathway of the direct reaction of carbohydrates with proteins to form a Schiff base, an Amadori intermediate and subsequent the formation of AGEs, is called the Amadori pathway, as shown in figure 5.[8, 29] Direct ROS-mediated oxidation of the side-chains of proteins results in protein carbonyl groups. The side-chains and backbone of these proteins are damaged.[8]

Oxidative stress is thought to be a very important mechanism in ageing and the pathogenesis of various diseases, such as cancer, neurodegenerative diseases, M. Alzheimer and complications in diabetes mellitus.[8]

*Antioxidative mechanisms*

Antioxidative mechanisms, as shown in table 1, are mechanisms which repair or prevent damage made by ROS, RNS and RCS. Several mechanisms are known, such as proteosomal degradation of damaged lipids and proteins, DNA repair mechanisms and chelation of metal ions in transport and storage proteins, such as haemoglobin, transferrin and albumin. ROS can be converted to less harmful molecules by different enzymes: superoxide dismutase (SOD) converts superoxide to hydrogen peroxide and catalase and glutathione peroxidase both decrease hydrogen peroxides. Vitamines A, C and E are also known as antioxidants. Vitamin A is able to scavenge singlet oxygen and vitamin C and E can reduce ROS to less harmful molecules.[26] There is evidence that vitamin B6 has anti-oxidative capacities.

| **Pro-oxidant** | **Antioxidant** |
|---|---|
| Metals:<br>- Decompartmentalization (not sequestrated, free redox-active metal ions)<br>- Overload | Metal sequestration:<br>- Albumin<br>- Transferrin, ferritin<br>- Hemopexin, haptoglobin |
| Inflammation:<br>- NADPH oxidase<br>- MPO | Antioxidant vitamins:<br>- Vitamin A<br>- Vitamin C<br>- Vitamin E<br>- *Vitamin B6?* |
| Drugs & xenobiotics:<br>- Alcohol<br>- Smoking | Small molecules:<br>- Glutathione<br>- Carnosine<br>- Uric acid |
| Environmental agents | Antioxidant enzymes:<br>- SOD<br>- CAT<br>- GPx |
| Hyperglycemia in diabetes mellitus:<br>- Glycation & AGE<br>- Polyol pathway | |

*Table 1:* Oxidative and antioxidative mechanisms. Modified from Baynes and Dominiczak[26]. AGE: advanced glycation end products, CAT: catalase, GPx: glutathione peroxidase, MPO: myeloperoxidase, SOD: superoxide dismutase.

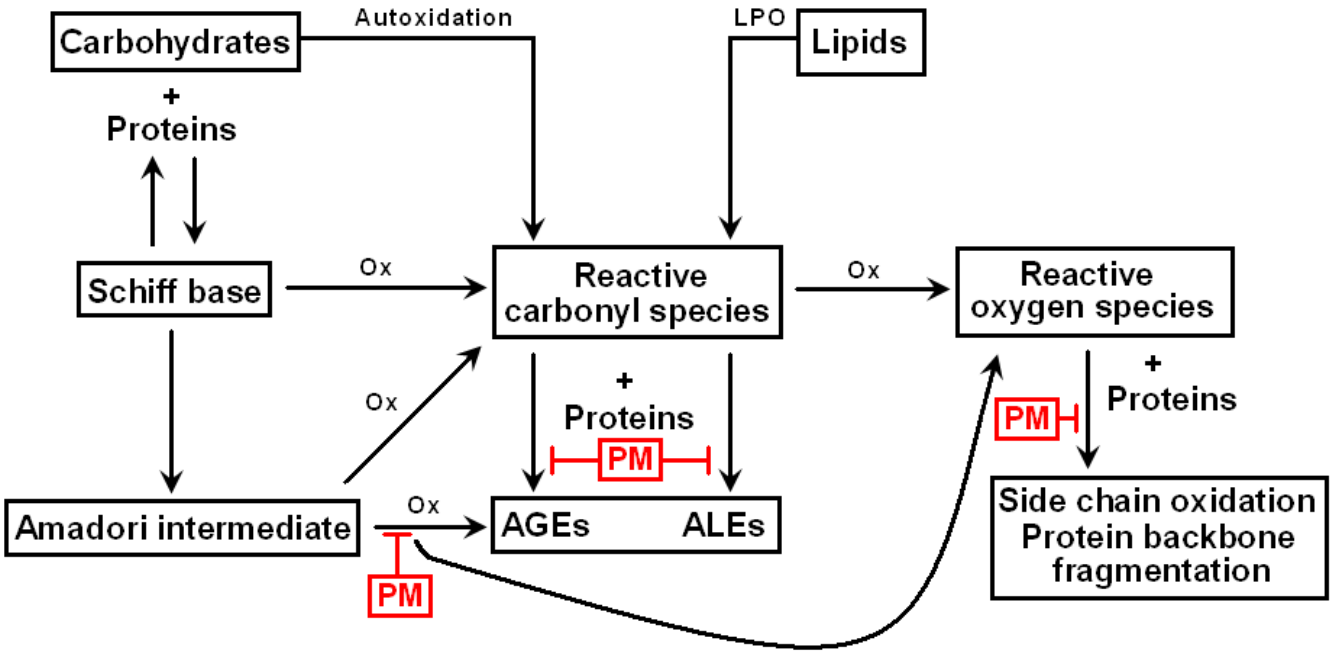


*Figure 5:* Overview of the anti-oxidative mechanisms of vitamin B6. Carbohydrates can react with proteins to a Schiff base, which is irreversibly converted to the Amadori intermediate. This pathway is called the Amadori pathway. Autoxidation of carbohydrates, peroxidation of lipids and oxidation of the Schiff base and Amadori intermediate from the Amadori pathway, result in the formation of reactive carbonyl species (RCS). Oxidation of the Amadori intermediate and the reaction of RCS with proteins, result in AGE and ALE formation. These reactions are inhibited by pyridoxamine by metal ion chelation and scavenging RCS, respectively. PM prevents ROS-induced protein side chain oxidation and backbone fragmentation by scavenging ROS. Modified from Voziyan et al[8, 40, 46]. AGEs: advanced glycation end-products, ALEs: advanced lipoxidation end-products, LPO: lipid peroxidation, Ox: oxidation, PM: pyridoxamine.

**The functions of vitamin B6 in oxidative stress**

The anti-oxidative properties of vitamin B6 and especially pyridoxamine have been reported in multiple studies. In in vitro and in vivo studies, oxidative stress was induced in several ways in human monocytic cells (U937)[30, 31], bovine endothelial cells (NM-1 cells)[32], yeast[33, 34] and rats[35, 36]. In all studies various vitamers of vitamin B6 were shown to suppress oxidative stress. PM is shown to be the most effective anti-oxidant[8, 34], but also PLP, PN and PMP were shown to have anti-oxidative capacities. Chumnantana et al[33] showed that PLP, PMP and PM are even more effective antioxidants than vitamin C. AGE and ALE formation were shown to be inhibited by vitamin B6[8, 29, 37-44]. Four different mechanisms that explain the anti-oxidative capacities of the B6 vitamers were hypothesized and investigated; they can directly scavenge ROS, scavenge RCS, chelate metal ions and increase or maintain GSH synthesis.

*Scavenging ROS*

A few studies showed that vitamin B6 can directly scavenge singlet oxygen[34, 45]. In a kinetic study, Ohta et al[45] found that pyridoxine reacts with singlet oxygen to hydroperoxide and endoperoxide. Bilski et al[34] found that PM, PLP and PL react directly with singlet oxygen. They showed that PM is the most effective scavenger at physiological pH. Possibly, the B6 vitamers and especially PM can scavenge also other oxygen radicals, such as hydroxyl and superoxide[8, 31, 43]. The hydroxyl group is shown to be important for the anti-oxidative function, probably because it can donate a hydrogen atom thereby reducing oxygen radicals.[8, 39, 42]

*Scavenging RCS*

Reactive carbonyl species, such as glyoxal (GO), methylglyoxal (MGO), glycolaldehyde (GLA) and 3-deoxyglycosone (3-DG), are formed during the oxidation of carbohydrates, amino acids, lipids and the Schiff base and Amadori intermediate formed in the Amadori pathway, as shown in figure 5. PM is shown to scavenge these RCS directly by binding to RCS.[8] GO and GLA are shown to form a Schiff base with PM, GOPM and GLAPM, by binding to both the hydroxyl group and aminomethyl group[46]. MGO is shown to bind only to the aminomethyl group to form MGOPM[47]. Also dicarbonyl compounds and other intermediates of lipid peroxidation are shown to bind to both groups[37]. Chetyrkin et al[48] showed that PM not only scavenges RCS by direct binding to them, but also that PM is involved in the cleavage of 3-DG, another RCS, thereby reducing it.

*Metal ion chelation*
Vitamin B6 is also suggested as a chelator of metal ions. As described previously, redox-active metal ions catalyze ROS production. Thus, by chelation of these ions, oxidative stress can be reduced. Endo et al[32] found that PN reduced oxidative stress in NM-1 cells induced by treatment with homocysteine and copper. They hypothesized that PN chelates the copper ions. PM is known to form complexes with copper and ferrous ions[49]. Voziyan et al[40] showed that PM does not form a complex with the Amadori intermediate, but inhibits its oxidation by chelation. They found that the hydroxyl group and aminomethyl group were necessary for complex formation of PM with metal ions, as shown in figure 6. Adrover et al[39] found that metal ions are more susceptible to complex formation with PM than the Amadori intermediate, indicating that PM inhibits oxidation of the Amadori intermediate by complex formation of metal ions with PM.

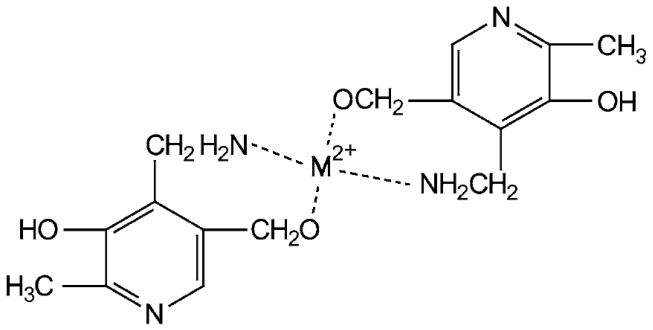


*Figure 6:* The aminomethyl- and hydroxyl-group of pyridoxamine (PM) are involved in complex formation of PM with metal ions ($M^{2+}$). Modified from Voziyan et al[12].

*GSH synthesis*
A few studies[30, 33, 36] suggested that the antioxidative effect of vitamin B6 is partially due to its effects on GSH synthesis in one-carbon metabolism. GSH can reduce hydrogen peroxide and lipid peroxides, as shown in figure 3. Decreased GSH levels due to oxidative stress were shown to be restored completely or partially by treatment with vitamin B6[30, 33, 35]. As described previously, vitamin B6 is a cofactor for cystathionine β-synthase and cystathionine γ-lyase, both enzymes involved in GSH synthesis. GSH prevents oxidation of thiol groups in the side-chains of amino acids. These studies hypothesized that vitamin B6 increases GSH levels by stimulation of the transsulfuration pathway of the one-carbon metabolism, thereby decreasing oxidative stress and its effects.[30, 33, 36]

In conclusion, vitamin B6 has antioxidative capacities and several mechanisms have been suggested, such as direct scavenging of ROS and RCS, chelating metal ions and increasing GSH synthesis.

## The role of vitamin B6 in immune system regulation

The immune system is a very complex defense system against invasion of foreign organisms and it can be divided into innate and acquired immunity. The innate immune system is a fast, but aspecific defense mechanism against foreign organisms in general. When the innate immune system does not succeed, the acquired immune system is activated in a couple of days and a specific defense reaction is accomplished. Vitamin B6 is thought to affect mainly the acquired immune system, but also the innate immune system. These systems cooperate closely, as shown in figure 7.

*Innate immune system* – The innate immune system consists of a humoral (non-cellular) and a cell-mediated part. Cells involved in innate immunity are for example neutrophils, macrophages and natural killer (NK) cells. *Neutrophils* and *macrophages* can completely surround foreign organisms, called phagocytosis, and degrade them. They present particles of these foreign organisms, *antigens*, on their cell membranes. Therefore, these cells are called *antigen presenting cells* (APCs). Antigen is presented on their cell membranes by specific receptors, called major histocompatibility complex (*MHC*) receptors. Antigen on MHC receptors can be recognized by cells of the acquired immune system. *NK cells* lyse cells infected with virus by excreting cytotoxic compounds. The ability to degrade cells by these cytotoxic compounds is called *cytotoxicity*. *Interferon* (IFN), a cytokine, mediates a defense reaction against viruses. The humoral part of the innate immune system consists of the complement system, interferon and other cytokines involved in immunity, called *interleukins* (ILs). The *complement system* is a cascade of activation of small molecules in serum, which can bind to foreign bodies and enhances its phagocytosis and degradation. Cells of the innate immune system enhance the acquired system by antigen presentation and secretion of interleukins.[50]

*Acquired immune system* – The main players of the cell-mediated acquired immune system are T- and B-lymphocytes. T- and B-lymphocytes are also called CD3- and CD19-positive cells, respectively, because they carry specific proteins on their cell membranes, called CD3 and CD19. T-lymphocytes can be divided in T helper (Th) 1 and 2 cells (CD4 positive) and cytotoxic T-lymphocytes (CTL, CD8 positive). Th-lymphocytes are activated by interaction of their T cell receptor with MHC receptors of APCs with antigen bound to it, as shown in figure 8. Activated T-lymphocytes will proliferate and secrete ILs. By excreting ILs, Th-1 cells can stimulate CTLs to proliferate and Th-2 cells can stimulate B-lymphocytes to proliferate.

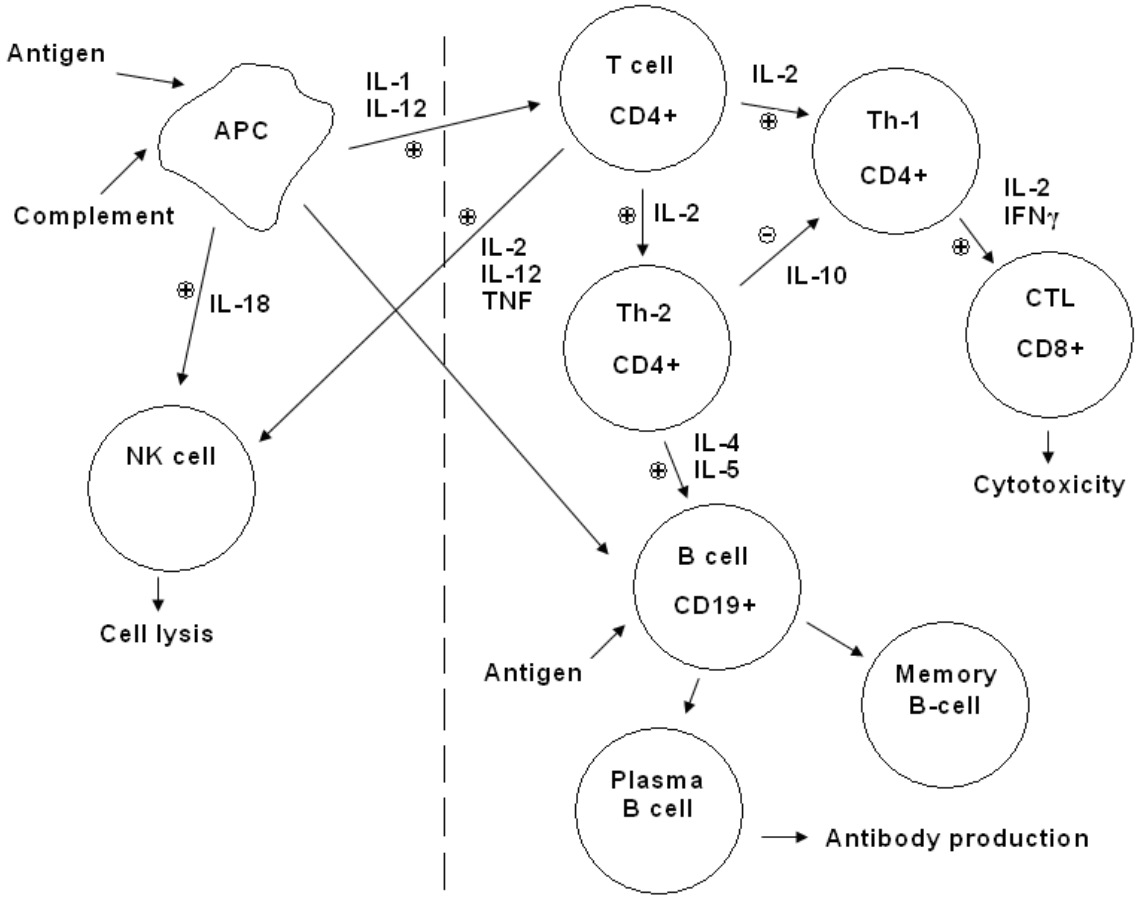


*Figure 7:* Overview of the immune system. When antigen is presented to APCs, APCs stimulate the cells of the innate and acquired immune system to differentiate and proliferate by secretion of interleukins. T-lymphocytes can differentiate to Th-1 cells, stimulating cytotoxicity of CTLs, or Th-2 cells, stimulating B cells to proliferate and produce antibodies. NK cells can lyse infected cells.[50] APC: antigen presenting cell, CTL: cytotoxic T-lymphocyte, IFN: interferon, IL: interleukin, NK cell: natural killer cell, Th: T-helper cell, TNF: tumor necrosis factor.

*CTLs* can, like NK cells, lyse foreign organisms by excreting toxic components, but they are directed to specific antigens. Stimulated *B-lymphocytes* will produce specific *antibodies*, immunoglobulins (Ig), the main components of the humoral part of the acquired immune system. Antibodies are antigen recognizing receptors which can bind to foreign particles and enhance degradation of these particles. A part of stimulated T- and B-lymphocytes will proliferate to memory cells. Therefore, during a second invasion of the same foreign organism, the acquired immune system can react faster by proliferation of these memory cells.[50]

Vitamin B6 is thought to influence the acquired cell-mediated and humoral immunity. Animal and human studies showed an association between vitamin B6 deficiency and impairment of several immune functions, such as the development of lymphoid organs, proliferation of T- and B-lymphocytes, cytotoxicity of T-lymphocytes, delayed type hypersensitivity reactions, antibody production and the production of several interleukins. Therefore, adequate levels of vitamin B6 are required for a normal immune response. Some studies found improvement of immune functions in vitamin B6 supplementation, indicating that vitamin B6 enhances an immune response, but in most studies the supplemented subjects were vitamin B6 deficient first. Only a few studies showed improvement of immune functions above normal levels in subjects treated or repleted with an excess of vitamin B6. In contrast, a few recent studies showed decreased vitamin B6 levels in inflammation, indicating that vitamin B6 has also anti-inflammatory properties[51-53].

The influence of vitamin B6 status on one-carbon metabolism, as shown in figure 2, is thought to be the underlying mechanism of the associations between vitamin B6 deficiency and impairment of immune functions.[7] As described previously, in vitamin B6 deficiency the synthesis of one-carbon units is most likely decreased. Therefore, the capacity of cells to synthesize nucleic acids and consequently DNA, RNA and protein could be decreased. An immune response is based on proliferation of immune cells and synthesis of proteins for proliferation, but also other proteins, such as antibodies and interleukins. Therefore, in immune responses an increased level of one-carbon units is required for DNA, RNA and protein synthesis and cell proliferation.[7]

Studies hypothesized that in vitamin B6 deficiency, cell proliferation in lymphoid organs and proliferation of (cytotoxic) T- and B-lymphocytes is reduced, because of reduced availability of one-carbon units for DNA, RNA and protein synthesis[5, 54-62]. These studies also suggested that the reduced availability of one-carbon units indirectly could account for decreased antibody and interleukin production.[55, 63]

Some studies[5, 54, 55, 57, 60, 64, 65] hypothesized another mechanism, which is investigated by Willis-Carr and St Pierre[66] and Chandra et al[67]. Thymic epithelial (TE) cells normally produce thymic factor, called thymulin. This factor is thought to stimulate differentiation of T-cell precursors in the thymus to mature functional T-lymphocytes. An in vitro study of Willis-Carr and St Pierre[66] showed a reduced ability of TE cell monolayers of rats treated with DPD and fed a vitamin B6 deficient diet simultaneously, to stimulate differentiation of T-cell precursors of rats fed a normal diet, compared to TE cell monolayers of untreated rats fed a normal diet. In contrast, they found a normal ability of TE cell monolayers of untreated rats fed a normal diet, to stimulate differentiation of T-cell precursors of rats fed a vitamin B6 deficient diet. These results indicate that vitamin B6 deficiency reduces the ability of TE cells to stimulate differentiation of T-cell precursors, possibly by affecting thymulin activity. Chandra et al[67] found a decreased thymulin activity in vitamin B6 deficient rats, compared to untreated rats on a normal diet. These studies indicate that in vitamin B6 deficiency, T-cell maturation is reduced by decreased thymulin activity.[67] Willis-Carr and St Pierre[66] hypothesized that in vitamin B6 deficiency the synthesis and/or release of thymulin is decreased, but they do not described the underlying mechanism.

Studies hypothesized that in vitamin B6 deficiency, decreased thymulin activity is responsible for the impairment of the development of lymphoid organs[55], lymphocyte proliferation[57], cytotoxicity of T-lymphocytes[54], antibody production[55] and interleukin production[5, 55, 57, 60, 64, 65]. They suggested that in vitamin B6 deficiency, there are less functional T-cells available, because of reduced T-cell maturation, for T-cell proliferation in lymphoid organs, proliferation of cytotoxic T-cells, stimulation of B-lymphocytes to produce antibodies and stimulation of T- and B-lymphoyctes to produce interleukins.[5, 54, 55, 57, 60, 64, 65]

In contrast, a few recent studies found decreased levels of vitamin B6 in inflammation and showed an inverse association between vitamin B6 and CRP levels[51, 52], indicating that vitamin B6 is important in an anti-inflammatory state and possibly has anti-inflammatory properties. A recent study of Yanaka[53] found an explanation for this suggested anti-inflammatory effect of vitamin B6. They found a reduced expression of serine protease inhibitor clade A member 3 (SPI-3) mRNA in rats, first fed a vitamin B6 deficient diet and then supplemented with pyridoxine, compared to rats fed only a vitamin B6 deficient diet. SPI-3 is a gene regulatory protein, which inhibits protein breakdown in inflammation thereby enhancing inflammation. Yanaka et al[53] showed that vitamin B6 can inhibit polyubiquitination of Iκ-B, a protein involved in the transport of NF-κB to the nucleus. NF-κB is a transcription factor which enhances transcription of proteins involved in inflammation, such as SPI-3, but also the transcription of cytokines, chemokines and adhesion molecules. Normally Iκ-B prevents transport of NF-κB to the nucleus thereby suppressing inflammation. Phosphorylation and polyubiquitination of Iκ-B induce proteosomic degradation of this protein, but vitamin B6 can inhibit in some way the polyubiquitination. Therefore, vitamin B6 prevents degradation of Iκ-B, thereby inhibiting transport of NF- κB to the nucleus and reducing transcription of proteins involved in inflammation. In that way, vitamin B6 enhances suppression of transcription of inflammatory proteins.

Salhany and Schopfer[68] and Namazi[69] suggested another mechanism for the anti-inflammatory properties of vitamin B6. Normally, T-lymphocyte proliferation is induced by interaction of T-cell receptors of Th-lymphocytes with MHC II receptors of APCs, on which antigens are presented. The CD4 protein on the surface of Th-cells stabilizes this interaction by binding to the MHC II receptor.

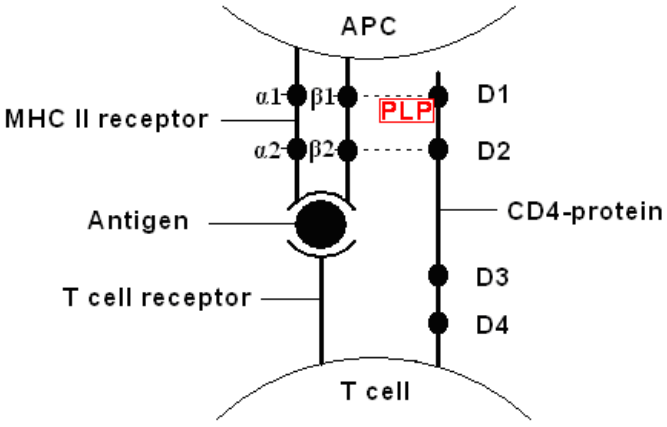


*Figure 8:* T-lymphocytes interact with APCs by binding of their T-cell receptor to antigen presented on MHC II receptors on the surface of APCs. The CD4 protein stabilises this binding by interaction of its D1 and D2 domains with the β2 and β1 domains of the MHC II receptor. PLP is shown to bind to the D1 domain of CD4, thereby covering the binding site for the β1 domain of MHC II and preventing stabilisation of the interaction of T-cells and APCs. Modified from Namazi[69]. APC: antigen presenting cell, MHC: major histocompatibility complex.

CD4 consists of four domains (D1-D4) and domain D1 and D2 normally interact with respectively the β2- and β1-domain of the MHC class II receptor, as shown in figure 8[68]. Salhany and Schopfer[68] showed that PLP can bind tightly to the D1 domain of the CD4 protein, thereby covering the binding site for the β2- domain of the MHC II receptor. Therefore, PLP can prevent stimulation of lymphocyte proliferation by APCs.

The researchgroup of Inubushi and Katunuma described another explanation for the anti-inflammatory properties of vitamin B6[70-72]. They showed a suppressed activity of cathepsin B and L in PN supplemented mice compared to non-supplemented mice. Cathepsins are proteases involved in the lysosomal breakdown of antigens needed for presentation of these antigens on MHC II receptors of APCs. Therefore, suppressed activity of cathepsins could inhibit breakdown of antigens for presentation thereby suppressing an immune response.[70-72]

Thus, adequate levels of vitamin B6 are important for maintaining the immune system and vitamin B6 deficiency impairs several functions of immunity, but possibly vitamin B6 also has anti-inflammatory properties. The associations between these functions and vitamin B6 status are described in more detail.

**Cell-mediated immunity**

*Lymphoid organs* – Lymphoid organs, such as the thymus, spleen, bone marrow and lymph nodes, are involved in the development and proliferation of T- and B-lymphocytes. In primary lymphoid organs, such as the thymus and bone marrow, T- and B-cells develop and maturate respectively, where as in secondary lymphoid organs, such as the spleen and lymph nodes, these cells are exposed to antigens, proliferate and interact with other T- and B-lymphocytes[50].

Vitamin B6 deficiency is shown to affect the development and function of lymphoid organs. Animal studies showed an association between vitamin B6 deficiency and lymphoid organ atrophy.[54-56, 73] After repletion of vitamin B6, Doke et al[55] found recovery of the lymphoid organ atrophy, but Ha et al[54] found no recovery at all. A study in pyridoxine supplemented patients with chronic renal failure, who are often vitamin B6 deficient, showed increased lymphocyte maturation in bone marrow.[74] Thus, adequate levels of vitamin B6 are required for normal development of lymphoid organs and for lymphocyte maturation.

Animal studies found that weights of spleen and thymus of rats and mice, fed a vitamin B6 deficient diet, were decreased compared to those of animals fed a normal diet[54, 55, 73]. Doke et al[55] and Ha et al[54] both confirmed a lower vitamin B6 level in blood and liver of these animals, respectively. Stoerck et al[73] found a depletion of lymphocytes in the thymus of rats fed a vitamin B6 deficient diet, a decreased differentiation between their thymic cortex and medulla and lymph node atrophy compared to rats fed a normal diet, but they did not confirm vitamin B6 depletion. A decreased number of lymphoid cells in thymus, spleen and thoracic duct has also been shown in mice fed a vitamin B6 deficient diet[55] and rats fed a vitamin B6 deficient diet and treated with DPD[56]. Robson et al[56] confirmed depletion of vitamin B6 in the animals, because they had skin abnormalities which could be attributed to vitamin B6 deficiency. In the study of Doke[55], the decreased weights of lymphoid organs and numbers of lymphocytes recovered after repletion of vitamin B6, but Ha et al[54] described no recovery of weights of spleen and thymus after repletion. Sjögren et al[74] found increased numbers of lymphocytes and monocytes in bone marrow in pyridoxine supplemented patients with chronic renal failure and haemodialysis. Vitamin B6 deficiency is common in these patients, but in this study no vitamin B6 levels are measured. They also found a decreased ratio of precursor cells/mature cells in bone marrow in these patients, indicating stimulation of maturation of lymphocytes by pyridoxine supplementation.

*Lymphocyte proliferation* – The effect of vitamin B6 status on proliferation of lymphocytes in rats, mice and humans has been well investigated. Animal and human studies showed that in vitamin B6 deficiency, T- and B-lymphocyte proliferation are decreased and that repletion of vitamin B6 recovers it partially, completely and in one study even above the baseline response[56-60, 75-78]. In healthy humans supplemented with pyridoxine, T-lymphocyte blood levels increased[79-81]. This indicates that adequate levels of vitamin B6 are required for normal lymphocyte proliferation responses, but possibly vitamin B6 also enhances it. It is unknown whether vitamin B6 status affects only B-lymphocytes though their action on T-lymphocytes, which normally stimulate B-lymphocyte to proliferate, or also B-lymphocytes directly.

Lymphocyte proliferation can be investigated in vitro by stimulating these cells in culture by T- and/or B-cell mitogens. In most studies, proliferation is measured by measuring DNA incorporation of labeled nucleotides. In all studies described here vitamin B6 deficiency and improvement of vitamin B6 status were proven in subjects on a vitamin B6 deficient diet and in vitamin B6 supplemented subjects, respectively.

An in vitro study of Scountzou et al[58] showed inhibition of T-lymphocyte proliferation in response to T-cell mitogens by medium addition of DPD in T-lymphocytes of healthy humans. In T- and B-lymphocytes of mice and rats fed a vitamin B6 deficient diet with and without DPD in drinking water, decreased proliferation reactions were shown in response to foreign lymphocytes[56, 75] and T-cell

mitogens[76]. In mouse T- and B-lymphocytes, the decreased proliferation recovered partially after PLP addition in vitro and PL injection in vivo[75].

In three studies, in humans supplemented with pyridoxine, numbers and percentages of T-lymphocytes and Th-lymphocytes in serum increased[79-81]. Cheng et al[79] found an increase of cytotoxic T-lymphocytes, but Folkers[80] showed an increased Th-cells/CTLs ratio, indicating that Th-cells are also stimulated by pyridoxine supplementation. In humans, the influence of vitamin B6 status on lymphocyte proliferation was investigated in elderly[57, 59], young women[60] and patients with chronic renal failure[77, 78]. Young women (n=7) first on a vitamin B6 deficient diet, were supplemented with pyridoxine[60]. Lymphocyte proliferation increased in the supplementation phase compared to the deficiency phase. In lymphocytes of vitamin B6 deficient elderly (n=15) supplemented with pyridoxine, proliferation increased in response to B- and T-cell mitogens compared to the proliferation of these lymphocytes before supplementation[59]. Meydani et al[57] found a reversible decreased number and percentage of lymphocytes in sera of healthy elderly on a vitamin B6 deficient diet (n=8) compared to these elderly on a normal diet. When they were on the vitamin B6 deficient diet, their T- and B-lymphocytes showed decreased proliferative responses to T- and B-cell mitogens, compared to the responses of their lymphocytes when they were on a normal diet. These responses also increased after repletion and even rose above baseline responses. Two studies investigated lymphocyte proliferation in patients with chronic renal failure[77, 78]. Most of these patients had a vitamin B6 deficiency (n=45) and compared to healthy controls (n=314), T-lymphocyte proliferation in response to T-cell mitogens was decreased[77], but improved after pyridoxine supplementation[77, 78].

*Cytotoxicity* – Cytotoxicity is the ability of for example NK cells and CTLs to degrade infected cells. Two studies found that in vitamin B6 deficiency the cytotoxicity of T-lymphocytes is impaired[54, 75]. The results of these two studies indicate that vitamin B6 plays a role in the cytotoxicity of T-lymphocytes.

The influence of vitamin B6 status on cytotoxicity of different cell types in mice has been investigated by Ha et al[54] and Sergeev et al[75]. Both described a decrease in cytotoxicity of T lymphocytes in mice fed a vitamin B6 deficient diet, compared to mice fed a normal diet. Ha et al[54] described in more detail a decreased primary and secondary cytotoxicity of splenic and peritoneal T lymphocytes in vivo in mice and in vitro against P815 mice tumor cells. They found no influence of vitamin B6 status on other types of cytotoxicity, such as antibody mediated cytotoxicity, cytotoxicity of natural killer cells and phagocytosis of macrophages[54].

*Delayed type hypersensitivity reaction and transplant rejection* – As described above, vitamin B6 deficiency is thought to suppress immune responses in several ways. In hypersensitivity reactions, such as allergy and delayed type hypersensitivity (DTH) reactions, suppression of these responses is desirable. Delayed type hypersensitivity is a T-cell mediated reaction; Th-1 cells recognize antigen presented on the surface of macrophages and initiate a hypersensitivity reaction by secreting IL-12 and tumor necrosis factor (TNF).[50]

Two old studies[82, 83] found that skin and systemic DTH reactions are suppressed in vitamin B6 deficiency and that after repletion these reactions recover to normal. This indicates that vitamin B6 is required for a normal DTH reaction. Transplant rejections are based on DTH reactions. In vitamin B6 deficiency, prolonged survival of skin homografts is shown in rats[84, 85]. Even skin homograft tolerance can be induced in vitamin B6 deficient rats by injection of splenic cells of the skingraft donor.[61, 62]

The research group of Axelrod and Trakatellis[82, 83] studied the influence of vitamin B6 deficiency on delayed type hypersensitivity (DTH) reactions in guinea pigs. Guinea pigs were immunized with Bacillus Calmette-Guévin (BCG), the attenuated live vaccine for tuberculosis. Skin[82] and systemic[83] DTH reactions were tested to the corresponding antigen, purified protein derivate (PPD). These reactions were depressed in guinea pigs treated with DPD and fed a vitamin B6 deficient diet, compared to untreated guinea pigs fed a normal diet. After vitamin B6 repletion DTH reactions recovered to normal. In vitro experiments showed that splenic cells of these vitamin B6 deficient animals were still sensitized to PPD. Conclusions from these results are that in vitamin B6 deficiency delayed type hypersensitivity reactions are depressed, but not the sensitization mechanism itself. Therefore, vitamin B6 must have affected the mechanism of DTH after sensitization[82, 83], but no concrete mechanisms are suggested. Possibly, because DTH reactions are mediated by T-lymphocytes, decreased proliferation of these cells by the mechanisms described previously, could inhibit DTH reactions[5].

In general, most transplant rejections are based on a DTH reaction[50, 86, 87]. Therefore, Axelrod et al[84] and Fisher et al[85] investigated the effect of vitamin B6 deficiency on the rejection of skin homografts in rats. Indeed, they both demonstrated a prolonged survival of skin homografts in rats fed a vitamin B6 deficient diet and treated with DPD simultaneously, compared to untreated rats fed a normal diet. After repletion of vitamin B6, in both studies these rats tolerated the skin homografts significantly longer compared to rats who were not deficient of vitamin B6 during the transplantation[84, 85]. This indicates that vitamin B6 plays a role in transplant rejections, presumably in the

DTH reaction causing transplant rejections. No explanation is given for the prolonged survival of skin homografts after repletion of vitamin B6.

Most likely, the suppression of immune responses by vitamin B6 deficiency, such as the DTH reaction in transplant rejections, is not specific. As described previously, other immune responses, such as T- and B-lymphocyte proliferation and cytotoxicity during infections, are also suppressed in vitamin B6 deficiency.[7, 84, 85] To achieve specific suppression of transplant rejection, Trakatellis and Axelrod[61, 62] investigated whether injection of splenic cells from donor mice can induce tolerance of their donated skin transplants in a different strain of vitamin B6 deficient mice. They found that mice fed a normal diet and injected with splenic cells of donor mice, did not tolerate skin homografts of these donor mice. In contrast, mice fed a vitamin B6 deficient diet, did tolerate the skin homografts after injection with those splenic cells. After vitamin B6 repletion, these mice still tolerated their transplants. This indicates that vitamin B6 deficiency enhances induction by splenic cells of immune tolerance of transplants. Only these two studies in mice are known, investigating the effect of vitamin B6 deficiency on transplant rejection. Therefore, more studies in animals and humans are required.

**Humoral immunity**

*Antibody production* – Binding of an antigen to an antigen receptor presented on the surface of B-lymphocytes stimulates these B-lymphocytes to proliferate and produce antibodies specifically directed to that antigen. Antibodies bound to their antigen can stimulate an immune response by binding to antibody receptors on T- and B-lymphocytes and other immune cells. They can be divided into five classes with different functions: IgM, IgG, IgA, IgE and IgD. As shown in figure 9, IgM is the first antibody produced in an immune response against an antigen.

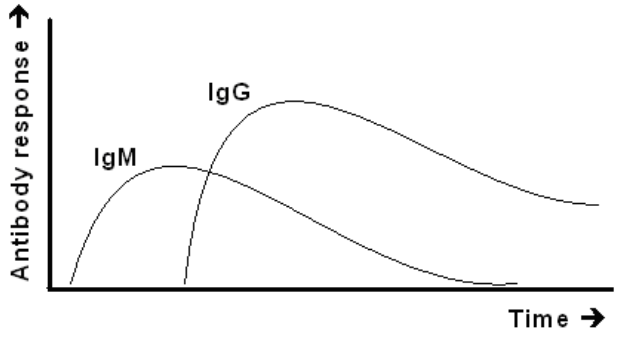


*Figure 9:* In an immune response, IgM is the first antibody produced. After a while, the plasma B-cells producing IgM switch to IgG production. Modified from Nairn[50]. Ig: Immunoglobulin.

This antibody is important in complement activation and is also presented on the surface of B-lymphocytes as antigen receptor. Later on in the same immune response, IgG is the predominant antibody, as shown in figure 9. B-lymphocytes which first produced IgM, can switch to IgG production. IgG is the main antibody present in serum and has four subclasses, $IgG_{1-4}$, with somewhat different functions. IgG is also important in antibody-mediated cytotoxicity. IgA is present in secretions, such as saliva, tears and mucus in the respiratory and gastrointestinal tract. In immune responses against parasites and in allergic reactions, IgE is the main antibody. IgE can bind to mast cells and basophils, the most important immune cells involved in allergy and parasite infections. The functions of IgD are relatively unknown, but it is, like IgM, present on the surface of B-lymphocytes as antigen receptor.[50]

Vitamin B6 deficiency is shown to impair antibody production in various animals, indicating that adequate levels of vitamin B6 are required for normal antibody production in response to various antigens[55, 63, 70, 73, 88, 89]. More studies are required to determine whether vitamin B6 also affects antibody production in humans[11, 80].

Antibody production has been investigated in relation to vitamin B6 status in studies in rats[73, 88], mice[55, 63, 70] and swine[89]. Almost all studies showed an impairment of antibody production in animals fed a vitamin B6 deficient diet in reaction to variable antigens, such as sheep red blood cells[73], influenza virus[88], human erythrocytes[89], Salmonella pullorum[89], dinitrophenylated ovalbumin (DNP-OVA)[55], Trichinella spiralis[63] and ovalbumin[70]. Frydas et al[63] also showed a decreased antibody production in animals treated with DPD compared to untreated animals. In contrast, Ha et al[54] found no impaired antibody-mediated cytotoxicity in mice fed a vitamin B6 deficient diet. Two studies[55, 89] showed full recovery of antibody production in vitamin B6 repleted mice and swine, respectively.

Three studies[55, 63, 70] made a distinction between classes of antibodies and showed decreased IgM, IgG and $IgG_1$ levels, produced in response to infection with the nematode parasite Trichinella spiralis, in DPD-treated mice compared to untreated mice[63], and a decrease of IgG, $IgG_1$ and $IgG_{2a}$ levels produced in response to (DNP-)OVA, in mice fed a vitamin B6 deficient diet compared to mice fed a normal diet[55, 70]. Doke et al[55] also found recovery of IgG levels after pyridoxine repletion.

Studies on IgE production in mice fed a vitamin B6 deficient diet showed contrasting results. Inubushi et al[70] found decreased IgE levels, produced in response to ovalbumin in these mice compared to mice fed a normal diet. In contrast, Doke et al[55] described increased IgE levels in vitamin B6 deficiency produced in response to DNP-OVA and normalization of these levels after pyridoxine repletion. A study of Inubushi et al[70] showed decreased IgE and $IgG_1$ levels in mice fed an excess of pyridoxine compared to mice fed a normal diet, indicating that vitamin B6 inhibits IgE production, thereby inhibiting allergic responses and IgE-mediated inflammation reactions against parasites.

Only two studies investigated the effect of vitamin B6 status on antibody production in humans. In a study of Hodges et al[11] two controls were on a normal diet and six men on a vitamin B6 deficient diet with (n=2) and without (n=4) DPD treatment. All men with the vitamin B6 deficient diet with and without DPD treatment had symptoms of vitamin B6 deficiency. In these vitamin B6 deficient men, who were immunized with typhoid and tetanus antigens, they found a slight, but not significant inhibition of antibody formation against both. Specific subclasses were not measured. Folkers et al[80] found unaffected serum IgG levels in healthy humans (n=9) supplemented with pyridoxine. These two small studies are not conclusive about whether vitamin B6 status affects antibody production in humans and so far, impaired antibody production is only shown in vitamin B6 deficient animals.

*Interleukin production* – Cytokines secreted by cells of the innate and acquired immune system are called interleukins (ILs). They can interact with their specific receptors on immune cells, thereby initiating, stimulating or inhibiting functions of these cells. IL-2, IL-6, TNFα and IFNγ are cytokines, which stimulate a Th-1 lymphocyte response. IL-4 and IL-10 are cytokines, which stimulate a Th-2 lymphocyte response and inhibit a Th-1 response. The relations of vitamin B6 status with IL-2, IL-4, IL-6, IL-10, TNFα and IFNγ production have been investigated.[50, 90]

Studies indicate that vitamin B6 deficiency impairs the production of cytokines, which stimulate a Th-1 response, such as IL-2, IL-6 and TNFα, but not IFNγ or the Th-2 response stimulating cytokines IL-4 and IL-10[55, 57, 60, 65, 90]. This indicates that normal levels of vitamin B6 are required for adequate communication between immune cells, especially in a Th-1 response.

IL-2 has been investigated in mice[55] and humans on a vitamin B6 deficient diet and in vitamin B6 deficient patients with Sjögren's syndrome[57, 60]. IL-2 is shown to stimulate T-lymphocyte proliferation. Doke et al[55] and Meydani et al[57] showed decreased IL-2 production in mice and elderly humans on a vitamin B6 deficient diet. Theses studies indicate that vitamin B6 deficiency decreases IL-2 production, thereby reducing stimulation of T-lymphocyte proliferation, but Kwak et al[60] and Tovar et al[65] both showed no change in IL-2 production in vitamin B6 supplemented young women and patients with Sjögren's syndrome, respectively.

A study of Frydas et al[90] in DPD-treated mice infected with the nematode parasite *Trichinella spiralis* showed inhibition of IL-6 and TNFα production and a slight inhibition of IL-4 production, but no inhibition of IL-10 and IFNγ production. However, Doke et al[55] reported an increase of IL-4 production in mice fed a vitamin B6 deficient diet, compared to mice fed a normal diet. Since IL-6 and TNFα are important in a Th-1 response and IL-4 and IL-10 in a Th-2 response, these results indicates that DPD inhibits the production of interleukins involved in a Th-1 response only. The increased IL-4 production in vitamin B6 deficiency found by Doke et al, supports the hypothesis that vitamin B6 also has anti-inflammatory properties.

## The role of vitamin B6 in carcinogenesis

The role of several B vitamins such as folate, vitamin B2 and vitamin B12 in carcinogenesis has been well investigated. The relation of vitamin B6 with carcinogenesis has also been well investigated in 'in vitro' studies, animal studies and human studies. Most of these studies concluded that there is an inverse relation between vitamin B6 and carcinogenesis, especially colorectal carcinogenesis. The exact mechanism is unknown, but a few mechanisms have been investigated and described in literature.

Carcinogenesis is the process of neoplasm formation. Neoplasms are tissue masses which grow uncoordinated and uncontrolled. They can invade surrounding tissue and metastasize to other tissues. Carcinogenesis is a process consisting of sequential DNA mutations causing disruption of defense mechanisms against neoplasm formation, thereby disturbing normal cell proliferation.[91] Normally, cell proliferation is well regulated with growth signals and inhibitory signals. DNA defects can be repaired by DNA repair systems and when that is not possible apoptosis occurs. In carcinogenesis, DNA damage occurs in genes involved in cell proliferation and repair mechanisms. Genes coding for growth promoting and growth inhibitory proteins are called proto-oncogenes and tumor suppressor genes, respectively. Mutation of these genes can lead to carcinogenesis. Because cancer cells need supply and removal of nutrients and waste products, angiogenesis is also a process involved in neoplasm formation.

**In vitro, animal and human studies**

In vitro studies showed a growth inhibition by vitamin B6 of different types of cancer cells in culture: hepatoma cells of rats[92] and human mammary[93] and melanoma cells[94-97]. Some studies[94, 95] showed an inhibition by pyridoxal and pyridoxine. Shultz et

al[97]however found growth inhibition by pyridoxal, but growth stimulation by pyridoxine. They gave no other explanation for the fact that PN stimulates growth than that they used a different cell line than DiSorbo et al and that their cells were not able to convert PN to PLP, the metabolic active form, because of a lack of the required enzymes, but that does not explain that PN stimulates cell growth. Maksymowych et al[96] showed that high levels of pyridoxine can kill human melanoma cells.

An early animal study by Gridley et al[98] showed growth inhibition by pyridoxine of fibrosarcomas in mice. Other animal studies demonstrated an inhibition of mammary carcinogenesis in rats by high levels of pyridoxine[99] and reduction of melanoma tumor volumes in mice by pyridoxine[96]. Komatsu et al induced colorectal cancer in mice and showed that these mice, when given a high pyridoxine diet, develop less colorectal tumors compared to mice given a low pyridoxine diet[100].

Most human studies on the relation of vitamin B6 with carcinogenesis focus on colorectal cancer, but also other types of cancer are investigated, such as lung, breast, renal, gastric, ovarian, prostate and pancreatic cancer. Most of them studied the association between nutrients involved in one-carbon metabolism, such as FAD, vitamin B6, vitamin B12, folate, methionine, homocysteine, cysteine and alcohol, and the cancer risk.

Vitamin B6 and the risk of colorectal cancer have been well investigated in prospective cohort studies[12, 101-105] and case-control studies[106-114]. Most studies showed an inverse association between vitamin B6 and the risk of colorectal cancer[12, 102-104, 106-108, 112-114], but some showed no association[101, 102, 105, 109-111] or a positive association (only in women)[101, 105]. Harnack et al[101] gave no explanation at all for the positive association that they found, but De Vogel et al[105] hypothesized that hypermethylation of gene promoters caused by vitamin B6, as described below, could account for the positive association. They gave no explanation why this positive association was only found in women, but in the study of Harnack only women were involved. Two recent meta-analyses[10, 115] reviewed these studies and both concluded that an inverse association between intake or blood levels of vitamin B6 and the risk of colorectal cancer is most likely.

In some studies the association of vitamin B6 and the risk of colorectal cancer was dependent on alcohol. In one study the inverse association was stronger in patients with low alcohol consumption[108], but in another study it was stronger in patients with high alcohol consumption[102]. Possibly, the association of vitamin B6 and the risk of colorectal cancer is alcohol-dependent, because alcohol influences one-carbon metabolism. Explanations for the stronger association in patients with high alcohol consumption are: alcohol inhibits absorption and stimulates renal excretion of vitamin B6 and folate[12, 102, 108, 116], it inhibits methionine synthase[12, 102] and lowers glutathione levels[12, 102]. Furthermore, Figueiredo et al[116] suggested that alcohol is converted to acetaldehyde, which is known to play a role in the colorectal carcinogenesis, possibly by altering DNA methylation. In contrast, no explanations for the stronger association in patients with low alcohol consumption, found by Le Marchand et al[108], were given.

Also in other types of cancer, such as lung cancer[117, 118], gastric cancer[119-121] and possibly also prostate cancer[122-125], an inverse correlation has been found between vitamin B6 and carcinogenesis. In breast cancer[126-132], renal cancer[133] and ovarian cancer[134, 135] however no association is found. In pancreatic cancer only Stolzenberg[136] showed an inverse correlation, but three other studies found no association at all[137-139].

**Mechanisms**

A few mechanisms have been suggested for the inverse association between vitamin B6 and carcinogenesis in these studies, but the exact mechanism is unknown. The most suggested mechanism is aberration of DNA methylation and synthesis by vitamin B6 influencing one-carbon metabolism, but other mechanisms described are alteration of gene expression and immune responses and suppression of oxidative stress, nitric oxide synthesis and angiogenesis.

*One-carbon metabolism*

The most suggested and studied mechanism of the association between vitamin B6 and carcinogenesis is the role of vitamin B6 in one-carbon metabolism. As described earlier, one-carbon metabolism is important for DNA synthesis and repair, DNA methylation, detoxification and protection against oxidation[12]. Therefore, in vitamin B6 deficiency these processes can be disturbed, enhancing carcinogenesis.

*DNA synthesis and repair* – As described previously, in vitamin B6 deficiency DNA and chromosome instability are possibly caused by an increased uracil misincorporation. DNA and chromosome instability can cause mutations, which can contribute to carcinogenesis.[12, 24, 104, 106-108, 110, 111, 113].

*DNA methylation* – As described previously, DNA methylation normally regulates gene transcription. Hypothetically, in vitamin B6 deficiency, DNA methylation is decreased because of less availability of SAM, the methyl group donor. DNA hypomethylation causes increased gene transcription.[12, 104-113]. Hypomethylation of gene promotors of proto-oncogenes and normally silenced harmful genes, such as viral insertions, repetitive elements, possibly causes increased transcription of those genes and can contribute to carcinogenesis.

Also hypomethylation of nuclear structures important for DNA stability can cause more DNA instability. In contrast, in almost all types of cancer, including colorectal cancer, an imbalance in DNA methylation is found; genome-wide DNA hypomethylation and gene promoter hypermethylation[25, 112, 140-142]. This imbalance is thought to cause an imbalance in transcription of proto-oncogenes and tumor suppressor genes. Often the promoters of tumor suppressor genes are hypermethylated, thereby inactivating these genes. In that way, hypermethylation also can contribute to carcinogenesis[25]. An association between vitamin B6 and hypermethylation is found[142, 143] and hypothetically, in vitamin B6 deficiency, hypomethylation can occur. But the exact relation of vitamin B6 and DNA hypo- and hypermethylation in cancer is unclear.

*Detoxification and antioxidation* – As described previously, the transsulfuration pathway of one-carbon metabolism and thereby GSH formation, is possibly inhibited in vitamin B6 deficiency. GSH is important in protection against oxidative stress and detoxification of carcinogenic compounds. Possibly, in vitamin B6 deficiency, DNA is less protected against oxidative stress and toxic compounds and thereby it contributes to carcinogenesis.[12, 114]

*Gene transcription*
Vitamin B6 is thought to alter gene transcription not only by altering DNA methylation, but also by direct inhibition of enzymes involved in gene transcription[9, 100, 144], such as DNA polymerase[145, 146], RNA polymerase[147, 148] and reverse transcriptase[149]. Vitamin B6 deficiency can lead to less inhibition of these proteins causing less inhibition of transcription of for example genes involved in cell proliferation.

PLP is also known as an inhibitor of transcription factors. For example, PLP can bind directly to transcription factors for the albumin gene and to steroid hormone receptors, which function also as transcription factors, thereby inhibiting gene transcription.[6]. Hypothetically, vitamin B6 deficiency could possibly account not only for less inhibition of transcription of the albumin gene, but also of for example proto-oncogenes. Thereby, carcinogenesis could be less inhibited in vitamin B6 deficiency. But according to this, PLP should also disturb gene transcription of other genes in normal cells, for example inhibition of transcription of tumor suppressor genes, thereby enhancing carcinogenesis. But there is no evidence in literature for this hypothesis. Thus, despite some authors suggested this mechanism as an explanation for increased carcinogenesis in vitamin B6 deficiency, it is unclear whether this mechanism plays a role or not. In conclusion, PLP can inhibit enzymes and other proteins involved in gene transcription by direct binding, but its role in carcinogenesis is unclear.

*Immune system*
As described earlier, vitamin B6 deficiency most likely contributes to the suppression of immune responses. Gebhard et al[150] speculated that carcinogenesis is suppressed by vitamin B6 through stimulation of an immune response. Tumor cells can produce proteins, which are not produced by normal cells, such as carcinoembryonic antigen in gastrointestinal cancer, and function as antigens, which can be recognized by the immune system and can induce an immune response against the tumor cells.[50] Some viruses can induce carcinogenesis as well. Gridley et al[98] showed suppression of herpes simplex virus type 2-induced carcinogenesis in mice supplemented with vitamin B6. Because in vitamin B6 deficiency, the immune system is suppressed, this could contribute to less suppression of carcinogenesis.

*Oxidative stress*
As described earlier, vitamin B6 has antioxidative properties. Oxidative stress contributes to carcinogenesis by damaging DNA[91]. Komatsu and Matsubara[9, 144] hypothesized that the antioxidative properties of vitamin B6 can be part of the suppression of carcinogenesis. They investigated this hypothesis by measuring oxidative stress markers in mice with induced colorectal cancer, fed a high pyridoxine diet[9]. They found a reduction in oxidative stress markers in mice with a high pyridoxine diet, compared to mice with a low pyridoxine diet. They also measured c-myc and c-fos expression, because some studies suggested that oxidative stress can elevate the expression of these proteins (Jang 1998, Crosby 2000). C-myc is a growth-stimulating transcription factor and c-fos is a protein involved in the post-transcriptional modification of transcription factors[91]. Upregulation of these proteins can cause growth stimulation, which can contribute to carcinogenesis. Komatsu and Matsubara[9, 144] found that mice with induced colorectal cancer, fed a high pyridoxine diet, did have lower expressions of c-myc and c-fos than mice fed a low pyridoxine diet[100]. Furthermore, a correlation between reduced oxidative stress markers and suppressed expression of these proteins was found. So, vitamin B6 indirectly inhibits oxidative stress induced c-myc and c-fos expression, which could contribute to the suppression of carcinogenesis by vitamin B6.

*Nitric oxide synthesis*
Nitric oxide synthases are enzymes involved in the production of nitric oxide (NO). One isoform, inducible NOS (iNOS), was investigated by Komatsu et al[9, 144] and Takahashi et al[151]. Takahashi et al showed elevated colonic iNOS expression in mice with induced colon tumors compared to mice without colon tumors.[151]

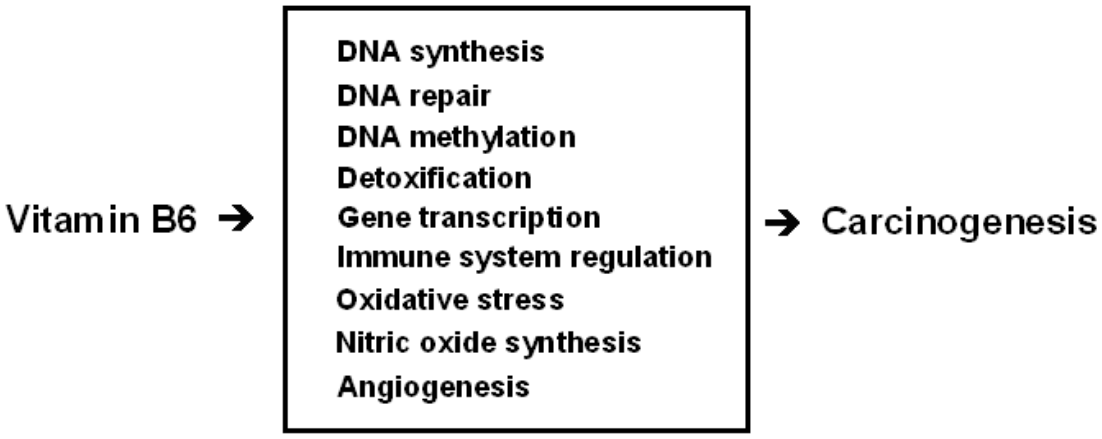


*Figure 10:* Overview of the effects of vitamin B6 status on carcinogenesis. Modified from Matsubara et al[144].

Komatsu et al found a reduced colonic iNOS expression in mice with induced colon tumors which were supplemented with vitamin B6 compared to mice with induced colon tumors, which were not supplemented.[9, 144]

NO is thought to play a role in carcinogenesis by damaging DNA, upregulating cyclooxygenase-2 and enhancing angiogenesis[9, 144, 151]. Cyclooxygenase-2 is an enzyme responsible for prostaglandin synthesis, which is upregulated in cancer and plays a role in carcinogenesis.[91] Komatsu et al[9, 144] hypothesized that vitamin B6 suppresses carcinogenesis by lowering iNOS expression and thus also NO synthesis.

*Angiogenesis*
The formation of new blood vessels is important in carcinogenesis. Matsubara et al[9, 144] investigated the effect of PLP and PN in vitro on the formation of micro blood vessels from cultured aortic rings of rats. They found that PLP and PN both inhibit this process. PLP inhibited angiogenesis stronger than PN in a dose-dependent way, but the mechanism is unknown.

In conclusion, most studies described above, found an increased cancer risk in vitamin B6 deficient subjects. Studies hypothesized that in vitamin B6 deficiency DNA synthesis, DNA methylation and DNA repair are disturbed, but vitamin B6 also enhances mechanisms which protect against carcinogenesis, such as decreasing transcription of genes involved in cell proliferation, detoxification of carcinogenic compounds and reduction of angiogenesis, oxidative stress, inflammation and nitric oxide synthesis, as shown in figure 10. Thus, vitamin B6 protects against carcinogenesis and in vitamin B6 deficiency mechanisms which normally protect against carcinogenesis are disturbed.

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# Part 2: The expression of the pyridoxal phosphate-dependent enzyme serine racemase in neuro 2a cells, glioma C6 cells and Hek 293T cells

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**Introduction** D-serine is an important co-factor of the NMDA-receptor in the brain, which is involved in the pathogenesis of multiple neurodegenerative diseases. D-serine is formed from L-serine by the pyridoxal phosphate dependent enzyme, serine racemase. To investigate the effect of vitamin B6 deprivation on D-serine concentration, at first we determined whether neuro 2A cells, glioma C6 cells or Hek 293T cells are appropriate cell lines by investigating whether serine racemase is expressed in these cells. **Materials and methods** Neuro 2A cells, glioma C6 cells and Hek 293T cells were grown and harvested. Western blot was executed with mouse anti-serine racemase as primary antibody and peroxidase conjungated goat anti-mouse as secondary antibody. **Results** Indefinite bands were visualized. Therefore, it is doubtful whether serine racemase is present in the cell lines. **Discussion** This experiment must be improved and repeated before further experiments can be done.

## Introduction

**D-serine**

For many years it was thought that only L-stereoisomers of amino acids had a function in physiology of mammalians[1, 2], but during the last decades some D-amino acids, D-serine, D-alanine and D-aspartic acid, are shown to be present in mammalian brain[1, 3].

D-serine is the enantiomer of L-serine, a non-essential amino acid[4]. L-serine can be converted into D-serine by the pyridoxal phosphate (PLP)-dependent enzyme serine racemase[3, 5], as shown in figure 1. Serine racemase is shown to be present in both astrocytes and neurons in the brain[5]. Co-factors of serine racemase are PLP, magnesium and ATP[3, 5].

D-serine is present in relatively high concentrations in the mammalian brain in the gray matter, the hippocampus, the amygdala and the anterior olfactory nucleus[1].

D-serine is shown to be a co-agonist of the N-methyl-D-aspartate (NMDA) receptor and binds to its 'glycine site', the site where glycine first was thought to bind. The NMDA receptor is located in the brain and functions as excitatory receptor for glutamate, an amino acid which functions as neurotransmitter[5]. Binding of glutamate and a co-agonist, such as D-serine are both necessary for activation of the receptor. Binding of D-serine decreases desensitization of the receptor, increases the affinity of the NMDA receptor for glutamate and increases its turnover by stimulating internalization[5]. Therefore, D-serine has a modulatory role in NMDA receptor function[5].

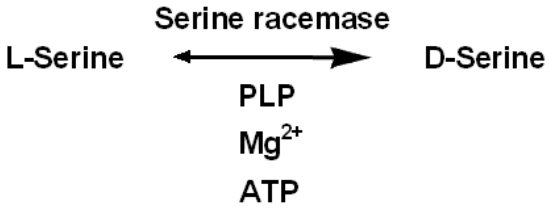


*Figure 1:* Chemical equation of the racemisation of L-serine into D-serine and vice versa by the PLP-dependent enzyme serine racemase. Serine racemase catalyzes the racemisation of L-serine in D-serine more than the other way around. Divalent cations, such as magnesium ($Mg^{2+}$), calcium ($Ca^{2+}$) and manganese ($Mn^{2+}$) and nucleotides, such as ATP, ADP and GTP, are co-factors of serine racemase and increase the production of D-serine[1-3, 5-8].

Activation of the NMDA receptor is important in several physiological processes, such as learning, memory, pain, synaptic plasticity and central nervous system (CNS) development[3, 5]. Especially, in the first weeks of life D-serine concentrations are high and they declined at infancy.

*Abbreviations*: APS, ammonium persulphate; BSA, bovine serum albumin; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal-bovine serum; PBS, phosphate-buffered saline; PDE, pyridoxine dependent epilepsy; PLP, pyridoxal 5'-phosphate; PNPO, pyridox(am)ine 5'-phosphate oxidase; SDS, sodium dodecyl sulphate; TBS-T, Tris-Buffered Saline with Tween 20; TEMED, tetramethylethylenediamine.

In 3-phosphoglycerate dehydrogenase deficiency, a disorder of L-serine synthesis with severe neurological abnormalities, D-serine levels in the brain are low at birth. D-serine levels were normal in a prenatally treated child with this disease. This indicates that D-serine is important for CNS development[2].

Hypostimulation of the NMDA receptor is thought to be involved in the pathological mechanism of schizophrenia[3, 5].

Hyperstimulation of the receptor is associated with multiple (neurodegenerative) diseases, such as stroke, epilepsy, polyneuropathies, chronic pain, Alzheimer's disease, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis[3, 5].
Therefore, changes in D-serine concentrations in the brain could contribute to the pathogenesis of these diseases[3].

**Serine racemase**
Because serine racemase is a PLP-dependent enzyme, vitamin B6 deficiency could affect D-serine levels in the brain. Early vitamin B6 deficiency is associated with certain neurological abnormalities, such as pyridoxine dependent epilepsy (PDE)[9, 10] and neonatal epileptic encephalopathy caused by pyridox(am)ine 5'-phosphate oxidase (PNPO) deficiency[10, 11]. No experimental data are known about the effects of vitamin B6 deficiency on D-serine levels. To investigate these effects, we need a cell line which produces D-serine, thus expresses serine racemase. Therefore, we investigated three cell lines, neuro 2A cells, glioma C6 cells and Hek 293T cells on the presence of serine racemase.

We have chosen these three cell lines, because they are (or seem like) neuronal cells and serine racemase is known to be expressed in neurons and astrocytes. The neuro 2A cell line is a mouse cell line derived from a neuroblastoma[12]. Neuroblastoma cells are derived from neuroblasts, precursor cells of neurons and glial cells. The glioma C6 cell line is a rat cell line of glial cells[12]. The Hek 293T cell line is a human embryonic kidney cell line, transformed with adenovirus 5 DNA[12]. This cell line seems very like neuronal cells and therefore we have tested it as well on serine racemase expression.

We expected to find serine racemase expression in glioma C6 cells and neuro 2A cells, because these are neuronal cell lines and serine racemase expression is described in literature in neurons and astrocytes (one of the glial cell types). Futhermore, expression of serine racemase in glioma C6 cells is described previously in literature by Sikka et al[13]. We expected to possibly find serine racemase expression in Hek 293T cells, because they have multiple characteristics of neuronal cells[14].

When we have found a cell line which expresses serine racemase and has serine racemase activity, we will deprive vitamin B6 levels in these cells and measure the effect on D-serine concentrations. At the end, we expected to find decreased D-serine synthesis in vitamin B6 deprivated cells, because PLP is a co-factor of serine racemase.

## Materials and methods

**Materials**
The neuro 2A, glioma C6 and Hek 293T cell lines were purchased from ATCC (American Type Culture Collection). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I supplement, 1% penicillin/streptomycin and trypsin were purchased from Invitrogen. The BCA protein assay kit was purchased from Pierce and complete V4, the protease inhibitor cocktail tablet, was purchased from Roche. Nitrocellulose membranes were purchased from Whatmann and the skimmilk from Sigma. The primary antibody, purified mouse anti-serine racemase antibody ($IgG_1$) was purchased from BD Biosciences and the secondary antibody, peroxidase conjugated goat anti-mouse antibody from Thermo Scientific. For the Western blotting the Precision Plus Protein Dual Color Standards purchased from Bio-rad was used. The ECL plus kit was purchased from Amersham Biosciences and the ECL procedure is executed using the ImageQuant LAS 4000 from Amersham Biosciences. β-mercapto-ethanol was purchased from Merck. Fetal-bovine serum (FBS), phosphate-buffered saline (PBS), Tris-HCl, ethylenediaminetetraacetic acid (EDTA), NaCl, sucrose, Acryl/bisacryl, sodium dodecyl sulphate (SDS), ammonium persulphate (APS), tetramethylethylenediamine (TEMED), electrophorese buffer, Tris-Buffered Saline with Tween-20 (TBS-T) were made in and/or used from our own laboratorium.

**Cell culture**
Neuro 2A cells (P34), glioma C6 cells (P44) and Hek 293T cells (P26), were grown at 37°C very well in 75 $cm^2$ flasks in DMEM supplemented with GlutaMAX™-I, penicillin/streptomycin and 10% FBS. After four days, cells were harvested. Therefore, cells were first washed twice with 10 mL cold (4°C) PBS, then treated with 1,5 mL trypsin. Cell suspensions were centrifuged for 5 minutes at 1300 rpm and cell pellets were resuspended in 10 mL cold PBS. Then, cell suspensions were centrifuged again at 1300 rpm for 5 minutes and resuspended in 1 mL cold PBS. These suspensions were centrifuged again at 13.000 rpm for 5 minutes at 4°C and cell pellets were frozen at -80°C. The whole experiment was executed in duplo.

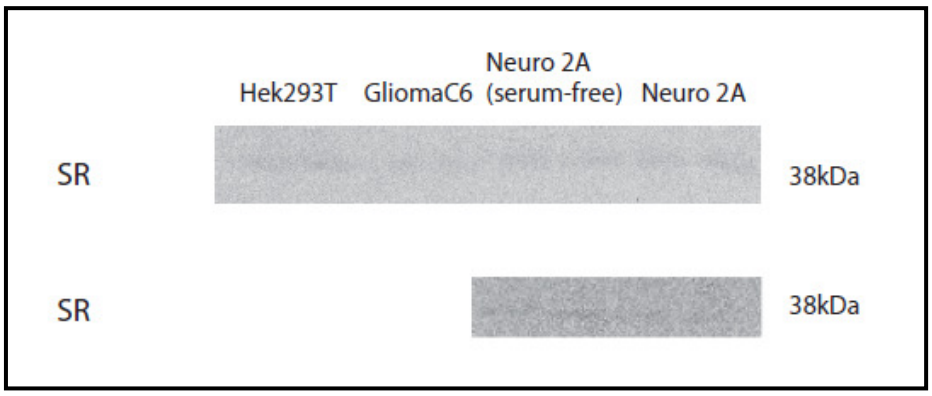


*Figure 2:* Western blots of serine racemase (~38 kDa) in Hek 293T cells, Glioma C6 cells, neuro 2A cells and neuro 2A cells treated with serum-free medium (neuro serum-free), visualized with ECL with 30 minutes illumination. The first western blot is one time stripped, but the second is not stripped.

**Differentiation of neuro 2A cells**
It is unknown whether serine racemase is expressed in undifferentiated neuronal cells. Therefore, 24 hours before harvesting, DMEM was replaced by serum-free medium for induction of differentiation of neuro 2A cells. When neuro 2A cells differentiate, their neurites will growth out. Tremblay et al[15] described that this method causes differentiation within minutes to hours.

**Protein assay**
To compare relative amounts of serine racemase on the Western blot film, equal amounts of protein must be loaded on gel. Therefore, a protein assay is executed first. Cell pellets were incubated with 250 µL lysisbuffer (50mM Tris pH 7,5; 5mM EDTA; 150 mM NaCl; 10% (w/v) sucrose) containing 10 µL protease inhibitor on ice for ten minutes. Samples were sonificated for 15 minutes and then 15 minutes centrifuged at 13.000 rpm at 4℃. 10 µL of the supernatant of the samples was incubated in triplo for 60 minutes at room temperature with 200 µL working reagent of the BCA protein assay kit. Absorbance was measured at 590 nm using the microplate reader from Biorad. The calibration curve was made with eight bovine serum albumin (BSA) standards with protein concentrations from 0 to 2000 µg/mL. Therefore, the working range of the protein assay was 125-2000 µg/mL

**Western blotting**
Samples were diluted and heated for 7,5 minutes at 95℃. 80 µg protein of each sample was loaded on a 10% SDS-polyacrylamide gel. Electrophoresis was performed at 60mA. Samples were blotted onto a nitrocellulose membrane in one hour at 300mA. The nitrocellulose membrane was blocked overnight at 4℃ with 5% skimmilk in TBS-T containing 0,05% Tween 20. The membrane was washed once with TBS-T and then incubated with the primary antibody, mouse anti-serine racemase (1:1000), for one hour at room temperature. Then, the membrane was washed three times with TBS-T for 5 minutes and incubated with the secondary antibody, goat anti-mouse (1:1000), for one hour at room temperature. After that, the membrane was washed again three times with TBS-T for 5 minutes and the protein was visualized with electrochemiluminescence (ECL) using the ECL-plus kit and the ImageQuant LAS 4000.

**Stripping**
The first time, the wrong antibody was used and therefore the membrane was stripped. The membrane was incubated at 50℃ in 50 mL stripping solution (0.85M Tris-HCl pH 6.7, 10% SDS, 14.34M β-mercapto-ethanol, demiwater). Then, the membrane was washed twice with PBS for ten minutes and twice with TBS-T for five minutes. The ECL procedure was done again to check whether all antibodies were stripped off the membrane. Then, the procedure described previously was followed for blocking, incubation with the primary and secondary antibody and ECL.

## Results

*Differentiation of neuro 2A cells*
Microscopically, we found no difference between neuro 2A cells which were 24 hours on serum-free medium compared to neuro 2A cells which were only on DMEM with FBS. Therefore, it is not likely that the neuro 2A cells on serum-free medium did differentiate.

*Protein assay*
Protein concentrations found were 3865 µg/mL (neuro 2A cells), 3984 µg/mL (neuro 2A cells 24 hours on serum-free medium), 2877 µg/mL (glioma C6 cells) and 5581 µg/mL (Hek 293T cells). These measurements were all far above the working range of the assay. Triplo's were very consistent.

*Western blotting*
The western blots of serine racemase in Hek 293T cells, Glioma C6 cells, neuro 2A cells and neuro 2A cells treated with serum-free medium (neuro 2A serum-free) are shown in figure 2. The second blot is from another experiment with neuro 2A cells treated the same as the neuro 2A cells of our experiment. The only differences are that on the second gel 40 µg protein was loaded and this blot was not stripped. This second blot was made due to a unintended switch of antibodies from another experiment.

Respectively, eight and four indefinite bands are visualized after 30 minutes illumination at ~37 kDa, the weight of serine racemase. Because of the obscurity of the bands, it is doubtful whether serine racemase is expressed in these cell lines. The western blot was also illuminated overnight with taking every hour a picture, but better bands could not be visualized.

Equal amounts of protein of each sample were loaded on the gel, but whether or not serine racemase is present, we cannot compare the relative amounts of the protein in these three cell lines, because of the obscurity of the bands.

## Discussion

We cannot conclude anything about the expression of serine racemase in neuro 2A cells, glioma C6 cells and Hek 293T cells, because the bands on the western blot film were obscure and we did not take any loading control, negative control or positive control along. Furthermore, it is uncertain whether the shown bands are exactly at 37kDa, because the ladder was not visible with ECL, but only with fluorescence. It was technically impossible to make an overlay of the bands and the ladder. Therefore, we compared the pictures of the bands and the ladder by hand, but we could not compare the bands with the ladder very well.

Previously, Sikka et al[13] did find serine racemase presence in glioma C6 cells. They used the same method as we did, except that they used another antibody, a polyclonal anti-mouse serine racemase, but our antibody is previously shown to be very effective. Possibly, our antibody is expired, because is it a few years old.

To improve the bands of the western blot, the experiment should be repeated with a positive control (i.e. cerebrum lysate of rats or mice), a negative control (milliQ, a purified protein other than serine racemase or theoretically a cell line in which there is shown before that there is definitely no serine racemase expressed), a loading control (i.e. tubulin or actin), higher protein and/or antibody concentrations and/or another antibody (i.e. the same as used by Sikka et al[13]. Protein concentrations could be increased by growing and harvesting more cells of each cell line. Furthermore, we expect that stripping decreased the intensity of the bands, because during the first experiment, bands on the film incubated with the wrong antibody were much more obvious, indicating enough protein was loaded on the gel.

Furthermore, the working range of the protein assay was 125-2000 µg/mL, but our samples had protein concentrations far above this range (2500-6000 µg/mL). Therefore, the measurements were far above the calibration curve and could be inaccurate. Nevertheless, that cannot be the only explanation for the obscure bands. Next time, sample could be diluted for a more accurate measurement.

It was not likely that the neuro 2A cells on serum-free medium did differentiate and therefore we cannot conclude anything about the expression of serine racemase in differentiated neuro 2A cells. Possibly, the cells were too long on serum-free medium (24 hours), because Tremblay et al[15] describes that the differentiation takes place within minutes to hours. Possibly, the lack of serum could affect the neuro 2A cells in another way.

To investigate whether serine racemase is expressed in differentiated neuro 2A cells, the differentiation could be repeated but then with a shorter period on serum-free medium or the cells could be differentiated in another way, for example by using forskolin, retinoic acid or 2,4-dinitrophenol[15]. Also differentiation could be checked not only microscopically, but also by measuring phosphorylation of proteins involved in differentiation, such as epidermal growth factor receptor, extracellular signal-regulated kinase 1/2 and Akt[15].

**Suggestions for further research**

When a cell line is found which expresses serine racemase, serine racemase activity must be determined, because when serine racemase is expressed but not active, it will be impossible to study the effect of vitamin B6 deprivation on D-serine synthesis. Serine racemase activity could be determined by comparing its activity in the normal situation and in the presence of an inhibitor, such as L-serine O-sulphate[16] (loss of function) or stimulator, such as $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, ATP, ADP or GTP (gain of function)[6, 7]. In addition, the cells with (the highest) serine racemase expression and activity, should be deprived of vitamin B6 to study the influence on D-serine concentrations.

Thus, no conclusion can be drawn on serine racemase presence in neuro 2A cells, glioma C6 cells and Hek 293T cells. This experiment must be improved and repeated before other experiments could be done and before conclusions can be drawn regarding the presence of serine racemase in these cell lines.

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