Vitamin B₆ and serotonin metabolism in neurological disorders of childhood

Submitted by:

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I, Emma Footitt confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that it has been indicated.

Signed………………………………….…..Date ………………………………...
Abstract

Pyridoxal 5’-phosphate (PLP) is the active form of vitamin B₆ in man where it functions as a cofactor for more than 140 enzyme catalysed reactions. Several inherited diseases characterised by seizures have been described which result in an intracellular deficiency of PLP; laboratory measurement of B₆ forms an important element in the diagnosis and monitoring of these disorders.

A review of PLP measured by HPLC in CSF from patients with neurological disorders showed that variance is greater than indicated by previous studies and the age-related reference limit was revised. This thesis also describes the metabolic disorders that may lead to PLP depletion and examines the relationship of CSF PLP to sulphite accumulation, medications and seizures in patient groups.

B₆ exists as six different vitamers and is catabolised to 4-pyridoxic acid for urinary excretion. An LC-MS/MS method was developed which could measure all vitameric forms in plasma. Its application to children with B₆ responsive seizure disorders showed that patients with inborn errors of metabolism have characteristic B₆ profiles which allow them to be differentiated from each other and control populations.

PLP is the cofactor for aromatic L-amino acid decarboxylase (AADC) which catalyses the final step in serotonin biosynthesis. This thesis tested the hypothesis that hyperserotonaemia observed in some patients with autism is related to an abnormality in this pathway by investigating the relationship between plasma B₆ vitamers, AADC activity and whole blood serotonin in a group of patients and controls. Plasma AADC activity was significantly reduced in autistic subjects; this is considered in the context of current biochemical and molecular understanding and its possible relevance to disease mechanisms is discussed.
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<tr>
<td>3Di</td>
<td>Developmental, Dimensional and Diagnostic Interview</td>
</tr>
<tr>
<td>3-MT</td>
<td>3-methoxytryrosine</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindolacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>5-HP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>5-methyltetrahydrofolate</td>
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<tr>
<td>α-AASA</td>
<td>α-amino adipic semialdehyde</td>
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<tr>
<td>AADC</td>
<td>Aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>ADOS</td>
<td>Autism Diagnostic Observation Schedule</td>
</tr>
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<td>Tetrahydrobiopterin</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CNV</td>
<td>Copy number variation</td>
</tr>
<tr>
<td>CP</td>
<td>Choroid plexus</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>d</td>
<td>deuterated</td>
</tr>
<tr>
<td>DDC</td>
<td>Dopa decarboxylase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and statistical manual of Mental Disorders</td>
</tr>
<tr>
<td>DTE</td>
<td>Dithioerythritol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
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<tr>
<td>ESE</td>
<td>Exon splicing enhancer</td>
</tr>
<tr>
<td>ESS</td>
<td>Exon splicing silencer</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<td>Gln</td>
<td>Glutamine</td>
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<tr>
<td>Gly</td>
<td>Glycine</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GTPCH</td>
<td>Guanosine triphosphate cyclohydrolase</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HFBA</td>
<td>Heptafluorobutyric acid</td>
</tr>
<tr>
<td>HIE</td>
<td>Hypoxic ischaemic encephalopathy</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>ICD</td>
<td>International classification of diseases</td>
</tr>
<tr>
<td>IDO</td>
<td>Indolamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence quotient</td>
</tr>
<tr>
<td>IUB</td>
<td>International Union of Biochemistry</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>L-dopa</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LNAA</td>
<td>Large neutral amino acids</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
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<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MoCoF</td>
<td>Molybdenum cofactor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NMDA</td>
<td>$N$-methyl-D-aspartate</td>
</tr>
<tr>
<td>Orn</td>
<td>Ornithine</td>
</tr>
<tr>
<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>P5C</td>
<td>Pyrroline 5-carboxylate</td>
</tr>
<tr>
<td>P6C</td>
<td>L-$\Delta$-piperideine-6-carboxylate</td>
</tr>
<tr>
<td>PA</td>
<td>4-pyridoxic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Pyridoxine dependent epilepsy</td>
</tr>
<tr>
<td>PEA</td>
<td>Phosphoethanolamine</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PNPO</td>
<td>Pyridoxamine 5’-phosphate oxidase</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PK</td>
<td>Pyridoxal kinase</td>
</tr>
<tr>
<td>PL</td>
<td>Pyridoxal</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PN</td>
<td>Pyridoxine</td>
</tr>
<tr>
<td>PNP</td>
<td>Pyridoxine 5’-phosphate</td>
</tr>
<tr>
<td>PM</td>
<td>Pyridoxamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMP</td>
<td>Pyridoxamine phosphate</td>
</tr>
<tr>
<td>PPi</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>PTPS</td>
<td>6-pyruvyltetrahydrobiopterin synthase</td>
</tr>
<tr>
<td>qBH₄</td>
<td>Quinonoid dihydrobiopterin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNI</td>
<td>Reference Nutrient Intake</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin reuptake transporter</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>Tau</td>
<td>Taurine</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TDO</td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TNSALP</td>
<td>Tissue non-specific alkaline phosphatase</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular Monoamine Transporter</td>
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Chapter 1
Introduction
1.1 THE METABOLISM, HOMEOSTASIS AND INBORN ERRORS OF VITAMIN B₆

1.1.1 Chemical structure of B₆ vitamers

Vitamin B₆ is a water soluble vitamin first described by György in 1934. The term vitamin B₆ is now used as a generic descriptor for all 3-hydroxy-2-methylpyridine derivatives that exhibit (in rats) the biological activity of pyridoxine (IUPAC-IUB). In man six B₆ vitamers exist differing only in the nature of the C4 and C5 substituent. The C4 carbon bears a hydroxymethyl group (-CH₂OH) in pyridoxine, an aldehyde group (-CHO) in pyridoxal, and an aminomethyl group (-CH₂NH₂) in pyridoxamine (Figure 1). All of these C4 variants can exist with the C5 substituent as a hydroxymethyl group or with this group esterified to phosphate (e.g. pyridoxal 5’-phosphate). In man pyridoxal 5’-phosphate is the active form, being a cofactor for more than 140 enzyme catalysed reactions.
Figure 1. Chemical structure of the B₆ vitamers and 4-pyridoxic acid
1.1.2 Reactivity of pyridoxal 5'-phosphate

Pyridoxal 5'-phosphate (PLP) has excellent electron sink properties making it a highly reactive and very versatile organic catalyst. The reactive aldehyde group of PLP can participate in a typical aldehyde reaction with many nucleophiles of both endogenous and exogenous origin. PLP undergoes a condensation reaction with amino groups to form a covalent Schiff base and it is this that initiates many of the reactions catalysed by PLP-dependent enzymes. PLP can also undergo a condensation reaction with hydrazines and sulphydryl compounds. The activated C3 carbon of Δ¹-pyrroline 5-carboxylate and Δ¹-piperidine 6-carboxylate can undergo a Knoevenagel condensation with the aldehyde group of PLP and this reaction is responsible for the increased B₆ requirement of patients with hyperprolinæmia type II and pyridoxine-dependent epilepsy due to antiquitin deficiency, respectively (1).

Much remains unknown about the physiological relevance of small molecule, non-enzymic interactions of PLP however the example of pyridoxine-dependent epilepsy due to antiquitin deficiency is a reminder that these non-enzymatic reactions of PLP can be extremely important.

1.1.2.1 Reactions of pyridoxal 5'-phosphate with lysine residues and N-terminal amino acids of proteins

**Enzymes**

PLP-dependent enzymes exist with PLP covalently bound via an imine bond to the ε-amino group of a lysine residue in the active site, forming an ‘internal aldimine’ or Schiff base (2). Disruption of this bond is the first step in the PLP dependent enzyme-catalysed reaction.

**Albumin**

At physiological concentrations the majority of PLP is bound to plasma proteins. Approximately 15% of this is bound via Schiff base formation to amino acids and peptides and the remaining 85% of PLP is bound via a Schiff base to serum albumin (3). Albumin appears to act as a reservoir, mediating PLP transport to tissues and protecting it from hydrolysis by phosphatases (4).
**Haemoglobin**

Both PLP and PL bind to haemoglobin and modify its oxygen-binding characteristics. PLP preferentially binds to the N-terminal amino acid of the β-chain of deoxy-haemoglobin and decreases oxygen-binding affinity, whilst PL is thought to bind to the N-terminus of the α-chain and produce an increase in oxygen affinity (5).

1.1.2.2 Reactions of B₆ vitamers with free radicals

There is evidence that, in some situations, B₆ vitamers can function as antioxidants, quenching singlet oxygen at a rate comparable to that of vitamins C and E (6;7). Details of these antioxidant mechanisms remain unclear (8) but pyridoxine has been found to be the most reactive of the vitamin B₆ species as an antioxidant and is twice as effective as PLP (6).

1.1.2.3 Reactions catalysed by pyridoxal 5’-phosphate-enzymes

PLP is the cofactor for a large number of essential enzymes and there are now more than 140 EC numbers assigned to distinct enzymatic reactions. The B₆-dependent enzymes acting on amino acid substrates belong to five of the total six enzyme classes as defined by the Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (9).

**Reactions involving amino acids**

PLP enzymes acting on amino acids have several common mechanistic and stereochemical features. All known PLP enzymes exist in their resting state with PLP bound covalently via an imine bond to the ε-amino group of a conserved active site lysine residue of the enzyme, forming the so-called ‘internal aldimine’ (Schiff base). The incoming, α-amino group of the substrate displaces the lysine ε-amino group from the internal aldimine, forming a new aldimine between the substrate and PLP (external aldimine / external Schiff base). The external aldimine is the common central intermediate for all PLP-catalysed reactions. During catalysis this external aldimine loses a proton to form a high-energy carbanion intermediate. The negative charge on the α-carbon is delocalised by resonance in the pi system of the cofactor which assumes a quinonoid form. In this way PLP stabilises anions generated at Cα
(10). Thereafter, the reactions diverge. Reactions at the $\alpha$-carbon atom of an amino acid substrate include transamination, decarboxylation, racemisation and elimination and replacement of an electrophilic group. Those occurring at the $\beta$- or $\gamma$- carbon atoms of amino acids include elimination or replacement (11). In all reactions PLP acts as an electron sink, temporarily storing electrons that are later used for the formation of new bonds (9) Figure 2.

Figure 2. Catalysis by pyridoxal 5’-phosphate enzymes. Formation of the quinonoid form of the external aldimine and carbanion at the C$\alpha$ carbon of the amino acid substrate

Reactions involving PLP-dependent enzymes are central to the synthesis and/or catabolism of many of the amino acids. Some of these amino acids and some of the products derived from them (e.g. dopamine, serotonin, histamine, D-serine, $\gamma$-aminobutyric acid and glutamate) function as neurotransmitters in the brain so it is not surprising that disturbed PLP homeostasis can lead to major neurological
manifestations such as intractable epilepsy. Other amino acid reactions catalysed by PLP provide markers for deficiency of the cofactor e.g. raised plasma homocysteine, increased urinary xanthurenic acid.

The majority of reactions catalysed by PLP-dependent enzymes result in regeneration of the cofactor. Thus the first step in a transamination reaction may generate pyridoxamine phosphate which is then converted back to PLP in the reaction with the oxo-acid that constitutes the second step of the two-step reaction. A number of decarboxylases and other B₆ dependent enzymes do, however, occasionally catalyse a half transamination reaction to produce an oxo-acid, pyridoxamine phosphate and the apoenzyme (12). Unless there is a moderately high concentration of PLP available to reconstitute the holoenzyme, this leads to inactivation. It is likely that pyridoxamine 5’-phosphate oxidase (PNPO) plays a salvage role in this instance, reconstituting the holoenzyme by converting pyridoxamine phosphate to pyridoxal phosphate. The extent to which pyridoxamine phosphate is generated by PLP dependent reactions \textit{in vivo} is uncertain.

1.1.2.4 Other pyridoxal 5’phosphate-catalysed reactions

Glycogen phosphorylase catalyses the breakdown of the storage polysaccharide, glycogen to yield glucose 1-phosphate. Unlike the majority of PLP dependent reactions where the aldehyde group is involved in catalysis, it is the phosphate group of PLP that participates in the catalytic role of glycogen phosphorylase (30,31).

1.1.3 Dietary sources of vitamin B₆

Vitamin B₆ is widely distributed throughout the plant and animal kingdom. Plants and most unicellular microorganisms can synthesise this vitamin, whereas humans and other animals must obtain it from external sources via intestinal absorption. Dietary sources rich in vitamin B₆ include vegetables, whole grain cereals, nuts and meat. The digestibility of vitamin B₆ from most plant products is on average 10% lower compared to animal products (13;14).

Isolated dietary deficiency of vitamin B₆ is rare. The vitamin is widely distributed in foods, although much of the vitamin B₆ in plant sources may be present as unavailable glycosides. Intestinal flora also synthesise large amounts, at least some
of which is absorbed. Absorption of B₆ vitamers may be impaired in coeliac disease (15), Chron’s disease and ulcerative colitis (16). In coeliac disease low levels of plasma PLP may persist despite treatment with gluten-free diet for many years (17).

1.1.4 Normal Physiology and Metabolic Pathways

Phosphorylated B₆ vitamers ingested in the diet must be hydrolysed to pyridoxal, pyridoxamine and pyridoxine by intestinal phosphatases prior to absorption at the enterocyte (18-21). Pyridoxine-glucoside from plant sources is hydrolysed to pyridoxine by pyridoxine-5’-β-D-glucoside hydrolase, and also the brush border membrane lactase, phlorizin hydrolase (22).

Initially absorption of these vitamers was thought to occur by simple diffusion across the brush-border of the jejunum and at high vitamin concentrations (possible in supplementation tests), passive transport does appears to dominate. Recent demonstration of a specialised carrier mediated system for pyridoxine in the small intestine suggests, however, that other mechanisms may also be important, although the molecular identity of this intestinal vitamin B₆ uptake system and its gene have yet to be elucidated (23). Free vitamin B₆ may also be synthesised in large amounts by the normal bacterial microflora of the human large intestine. This is thought to be taken up by colonocytes via a recently identified specific carrier-mediated mechanism (24).

Once absorbed pyridoxal, pyridoxamine and pyridoxine are re-phosphorylated and ‘trapped’ in the enterocyte. They must be dephosphorylated by plasma membrane phosphatases before being released into the portal vein and transported to the liver. Here the unphosphorylated B₆ vitamers are taken up rapidly by the hepatocytes and are phosphorylated by pyridoxal kinase (25). Pyridoxine phosphate and pyridoxamine phosphate are then converted to pyridoxal phosphate by cytosolic pyridox (am)ine-5’-phosphate oxidase (PNPO) (Figure 3).
Figure 3. The interconversions of vitamin B₆ in man
Figure 4. Catabolic pathways of pyridoxal 5’-phosphate to 4-pyridoxic acid

Approximately 10% of total body PLP is in the liver (25) and when formed, some PLP is transferred to PLP-dependent apoenzymes. The remaining PLP is released from the liver into the circulation bound primarily to the lysine-190 residue of albumin and forms approximately two thirds of total plasma vitamin B₆ with lesser amounts of pyridoxal, pyridoxine and pyridoxamine. Any unbound pyridoxal in the hepatocyte is oxidised by aldehyde oxidase to form 4-pyridoxic acid, the metabolic end product of PLP (26) (Figure 4). An NAD-dependent aldehyde dehydrogenase has also been reported to catalyse this reaction (27). 4-Pyridoxic acid is then released into the plasma and is excreted in the urine. About half of the normal dietary intake
of vitamin B₆ is excreted as 4-pridoxic acid with urinary excretion largely reflecting recent intake of the vitamin, rather than overall nutritional status (28).

PLP is unable to cross cell membranes and delivery of the active cofactor to tissues, therefore requires hydrolysis of circulating PLP to pyridoxal by the ecto-enzyme, tissue non-specific alkaline phosphatase. The major factor that determines plasma PLP levels appears to be the balance that is struck between liver formation and its breakdown by the alkaline phosphatase enzyme (28). Pyridoxal that is released close to the surfaces of cells is then used as needed by tissues and any excess pyridoxal enters the general circulation and is taken up by the liver where it is degraded to pyridoxic acid (28).

Only the unphosphorylated forms of vitamin B₆ are able to cross the blood-brain barrier probably by facilitated diffusion, mostly at the choroid plexi (CP). The CP then ‘traps’ PLP via pyridoxal kinase and, like the liver, can readily release it (29). Once within the CSF and extracellular space of the brain, the B₆ vitamers must be dephosphorylated so that they can enter brain cells. They are then metabolically trapped being re-phosphorylated by pyridoxal kinase. Pyridoxine and pyridoxamine phosphate are then oxidised by PNPO to form the active cofactor, PLP(30).

Excessive concentrations of the phosphorylated B₆ vitamers are dephosphorylated and transported out of brain cells (31). PNPO is inhibited by PLP, suggesting that this enzyme may be under control by feedback inhibition (32;33) but the regulation of PLP concentration in the brain is likely to be complex. Evidence suggests that raising the plasma concentration of the non-phosphorylated forms of vitamin B₆ decreases the relative amount entering brain cells due to proportionately less carrier-mediated flux through the blood brain barrier and more importantly, saturation of pyridoxal kinase in the brain, thereby decreasing the amount phosphorylated and retained. At low plasma concentrations, relatively more is accumulated by brain, thus tending to maintain brain B₆ cofactor levels (29).

1.1.4.1 Salvage Pathway

Besides the formation of PLP from the vitamins pyridoxine, pyridoxamine and pyridoxal supplied from ingested nutrients, PLP in mammalian cells can also be formed by recycling the cofactor from degraded enzymes in a ‘salvage pathway’.
This pathway for the synthesis of PLP is known to exist in many organisms and is responsible for the interconversion of the six different forms of vitamin B₆ and maintaining PLP homeostasis. A kinase converts pyridoxine, pyridoxamine and pyridoxal to their corresponding phosphorylated forms PNP, PMP and PLP, respectively. PNP and PMP can then be oxidised by PNPO to also form PLP. The phosphorylated forms can then be hydrolysed by phosphatases thereby restoring the free vitamers (34).

1.1.5 Homeostasis

Considering the large number and variety of reactions in which PLP is involved, homeostatic mechanisms within the cell as well as in plasma and cerebrospinal fluid (CSF) are of critical importance. The concentration of plasma B₆ vitamers is controlled by dietary supply and metabolic interconversion, amongst other factors. However, whilst the major pathways of vitamin B₆ metabolism are well established, the mechanisms by which PLP homeostasis is achieved are not yet fully understood.

Studies of dietary pyridoxine depletion (1.75 μmol/day for 6 weeks) and supplementation (0.98 mmol/day for 6 weeks) provide an insight into B₆ homeostasis in man, suggesting that the conversion of PLP to pyridoxic acid is subject to homeostatic regulation. Coburn et al. (35) demonstrated that urinary excretion of pyridoxic acid rapidly adjusts to approximate pyridoxine intake and although plasma and erythrocyte PLP also vary to reflect intake, muscle concentrations of PLP were not significantly altered. Similar results are reported in rats fed excess pyridoxine where dietary intake did not affect PLP concentrations in muscle, liver or brain however plasma levels of pyridoxal (PL) and 4-pyridoxic acid (4-PA) were significantly increased (36).

Concentration of B₆ vitamers and pyridoxic acid in plasma are in the order PLP>PA>PL>PN with much lower levels for PNP, PMP or PM (37). The fact that plasma PM and PMP levels are much lower than the other B₆ vitamers is in keeping with the observation that PM and PMP are not released from liver cells. In contrast, PMP and PLP are the major B₆ forms found in erythrocytes, the body tissues and in their mitochondria (25).
It appears that no single mechanism tightly regulates the cellular concentration of PLP; instead multiple different processes are likely to exist. During periods of vitamin B\textsubscript{6} sufficiency, factors that regulate PLP include the degree of binding to cellular proteins, transport of precursors and phosphatase activity. PNPO also plays a role in regulating PLP formation by negative feedback inhibition whereas pyridoxal kinase plays an intracellular compartmentalisation (‘trapping’) role because pyridoxal (but not PLP) is diffusible across the cell membrane (30;38;39). The intracellular content of hepatocytes is closely regulated preventing excess PLP accumulation with build-up of precursors. In plasma, however, the major B\textsubscript{6} vitamers are PLP and pyridoxal (37). Studies have shown that the liver is the sole organ responsible for the formation of plasma PLP whilst pyridoxal in plasma may derive from multiple organs. Human erythrocytes can convert pyridoxine into pyridoxal and release this B\textsubscript{6} vitamer and it is also known that the hepatocytes can release pyridoxal (as well as PLP and pyridoxic acid) into the circulation (37).

Various physiological and pathological conditions including pregnancy, fasting, myocardial infarction and certain drugs are known to lead to alter the normal equilibrium between the various B\textsubscript{6} vitamers (40). It should be borne in mind that a decrease in the concentration of plasma PLP may be accompanied by an equivalent increase in the level of PL, as a result of which the total amount of vitamin B\textsubscript{6} remains unchanged.

1.1.6 Circadian rhythm of pyridoxal kinase and pyridoxal 5’-phosphate

The homeostatic regulation of plasma PLP is achieved by various mechanisms as described above (Section 1.1.5) and animal studies suggest that in part, PLP levels are maintained by circadian clock controlled transcription factors. The PAR bZip (proline and acidic amino acid-rich basic leucine zipper) transcription factors DBP (albumin D-site-binding protein), HLF (hepatic leukaemia factor) and TEF (thyrotroph embryonic factor) show very high sequence conservation in mammalian evolution reflecting their important physiological functions. The striking feature of these three transcription factors is their robust circadian rhythm which is more pronounced in the periphery than in the central nervous system. All three factors are also expressed in a cyclic fashion in the suprachiasmatic nucleus; the major circadian pacemaker in mammals.
Recent work has shown that mice deficient in all three PAR bZip proteins are highly susceptible to spontaneous generalised and audiogenic seizures that may be lethal (41). Transcriptome profiling has revealed pyridoxal kinase (the enzyme responsible for conversion of B6 vitamers into the active cofactor PLP) as the primary target gene for these transcription factors. In support of this, decreased levels of PLP and the neurotransmitters serotonin and dopamine (likely to be causally related to seizure activity) are also observed in the triple knock-out mice.

Therefore, in mice, pyridoxal kinase is a clock-controlled gene and by controlling pyridoxal kinase, PAR bZIP transcription factors appear to play a vital role in the fine-tuning of PLP production and neurotransmitter homeostasis in the brain. At a physiological level the main function of circadian rhythm is to optimise metabolism and energy utilisation for sustaining life processes and it is probable that the oscillation of hepatic pyridoxal kinase expression contributes to the cyclic activity of enzymes involved in amino acid and glycogen metabolism. In contrast the almost invariable pyridoxal kinase expression in the brain is probably essential for the delicate regulation of neurotransmitter metabolism via PLP where only subtle changes may result in epileptic seizures (41).

Although studies of PLP circadian rhythm in man have not yet been undertaken, it will be interesting to explore the role of PLP as an enzyme regulator and translation into clinical practice will be important. Knowledge of PLP homeostatic mechanisms will not only broaden understanding of vitamin B6 metabolism but will allow tailoring of medical treatments where long term PLP therapy may be optimised by emulating normal physiological patterns.

1.1.7 Enzymes involved in interconversions of B6 vitamers

The enzymes involved in B6 vitamer interconversion play an important role in regulating the intracellular and body fluid concentrations of PLP. A dietary excess of vitamin B6 does not accumulate as PLP but is converted via phosphatase(s) and aldehyde oxidase(s) / dehydrogenase(s) to pyridoxic acid. Alongside these, pyridoxal kinase and PNPO also play important roles in homeostasis.
1.1.7.1 Phosphatases

Several phosphatase enzymes have been proposed to be involved in PLP regulation. The alkaline phosphatase enzymes (EC 3.1.3.1) can hydrolyse both PLP and PMP however they are not specific for vitamin B₆, most of them demonstrating broad substrate specificity for phosphomonoesters (42). Located on the outer surface of cell membranes, this group of ectoenzymes influence extracellular concentrations of PLP compared to the soluble phosphatases (with acid or neutral pH optima) which are thought to be involved in the regulation of intracellular PLP concentrations (43). These cellular phosphatases play an important role in the regulation of tissue PLP levels by rapidly hydrolysing free PLP when the amount exceeds the binding capacity of apoproteins. The pyridoxal formed can then readily pass out of the cell, across the cell membrane (44).

Some phosphatases do appear to be more specific for phosphorylated B₆ compounds. An acid phosphatase that may be specific for phosphorylated B₆ compounds has been partially purified from mouse liver (45) and a PLP phosphatase (EC 3.1.3.74) has been purified from human erythrocytes that appears to only hydrolyse phosphorylated B₆ compounds at high catalytic efficiency (46). More recently another phosphatase enzyme (the PHOSPHO2 gene product) has also been identified which shows a high specificity towards PLP (47).

Alkaline phosphatases

The alkaline phosphatases (EC 3.1.3.1) are a large family of enzymes that are found in many organisms (48;49). They catalyse the hydrolysis of phosphomonoesters with release of inorganic phosphate and an alcohol (49). These enzymes are glycoproteins which function as ectoenzymes and are anchored to cell membrane lipid bilayers via a glycosyl-phosphatidylinositol (GPI) anchor (49;50). In human tissues all forms of alkaline phosphatase (ALP) are primarily bound to the external surface of cells but ALP is also present in the circulation as an anchorless homodimer where the ALP isoenzymes are thought to reflect their source in specific organs.
Tissue non-specific alkaline phosphatase

TNSALP or liver/kidney/bone alkaline phosphatase is on chromosome 1p36.12. Much has been learned about the role of this enzyme in man through studies of hypophosphatasia which is an inherited deficiency of tissue non-specific alkaline phosphatase (TNSALP). This disorder of bone mineralisation is characterised by bony changes resembling rickets or osteomalacia and by the deficient activity of TNSALP in all tissues. In the absence of TNSALP, phosphorylated metabolites such as phosphoethanolamine (PEA) and pyrophosphate (PPI) are characteristically elevated and similarly there is a significant increase in levels of circulating PLP(51). This observation suggests that TNSALP is essential in vitamin B₆ metabolism and that PLP serves, alongside other endogenous phosphorylated compounds, as one of the physiological substrates for TNSALP.

TNSALP appears to act as an ectoenzyme to regulate extracellular but not intracellular levels of PLP (51) and it has been proposed that TNSALP is needed to dephosphorylate PLP to membrane-permeable forms of vitamin B₆ that can freely diffuse across cell membranes to then be rephosphorylated and used as a cofactor by various cellular enzymes.

Consistent with this, in TNSALP homozygous mutant mice, a greatly reduced concentration of PLP is found in the brain which may explain the lethal seizure disorder seen in these animals (52). Two neonates with perinatal hypophosphatasia have recently been described (53) who suffered from severe epileptic encephalopathy and had cerebrospinal fluid neurotransmitter changes indicating a functional deficiency of aromatic aminoacid decarboxylase (AADC) secondary to PLP depletion.

TNSALP is ubiquitous, being expressed in a variety of tissues during embryonic development and through postnatal and adult life (54;55). TNSALP has also been shown to be highly expressed in the placenta during the first trimester of pregnancy (56). In the postnatal brain, TNSALP is expressed in several cell types, including those found in capillaries, dura and choroid plexus (57).
**Pyridoxal 5'-phosphate phosphatase**

Human erythrocytes have at least two forms of PLP (PNP) phosphatase activity; an alkaline PLP (PNP) phosphatase activity that is associated with the stromal fraction, and is probably tissue-non-specific alkaline phosphatase (EC 3.1.3.1) (which is present in the plasma membranes of virtually all tissues) and a PLP phosphatase (EC 3.1.3.74) which is present in the soluble fraction of the erythrocyte (46). This phosphatase probably plays an important role in the hydrolysis of PLP to pyridoxal in erythrocytes and is located on chromosome 22q12.3. Erythrocytes rapidly take up pyridoxine and convert it to PLP. Free PLP that is not bound to protein, can then be hydrolysed by the PLP phosphatase to pyridoxal. Pyridoxal can then re-enter the plasma and be taken up by other tissues. This is an important source of PLP for those tissues that have very low PNPO activity and probably plays an important role in the regulation of vitamin B₆ metabolism (42;58).

**PHOSPHO2**

PHOSPHO2 is a putative human phosphatase which shares approximately 40% sequence identity with human PHOSPHO1, a phosphoethanolamine / phosphocholine phosphatase thought to be involved in the generation of inorganic phosphate for bone mineralisation (47). PHOSPHO2 has high specific activity towards PLP, in contrast to its activity towards phosphoethanolamine and phosphocholine which is poor and is believed to be a cytosolic protein and has a wide tissue expression. PHOSPHO2 is located on chromosome 2q31.1 and its importance in human physiology and potential role in the regulation of vitamin B₆ metabolism has yet to be determined.

**1.1.7.2 Pyridoxal kinase**

Evidence suggests that most eukaryote organisms contain a single pyridoxal kinase, coded by a pdxK gene (59) the sequence of which is highly conserved across different species (60). In humans pdxK has been localised to chromosome 21q22.3 and encodes a protein that has 312 amino acid residues. Individuals with Trisomy 21 (Down’s syndrome) are known to have altered B₆ metabolism (61) and have been reported to have elevated pyridoxal kinase activity (62). Pyridoxal kinase is
ubiquitously expressed in humans; the main site of enzyme activity is in the liver but it is also present in the testes (63;64).

Pyridoxal kinase belongs to the ribokinase superfamily and it is a dimeric enzyme with one active site per monomer (65). Whilst it is generally accepted that pyridoxal kinase phosphorylates all of the non-phosphorylated $B_6$ vitamins the human pyridoxal kinase favours pyridoxal as the substrate, whilst the usage of PN and PM as substrates decreases its activity by up to 70% (66).

The erythrocyte pyridoxal kinase activity of African-Americans is approximately 50% lower than that of persons with European ancestry. This racial difference is tissue-specific, with leucocyte and skin fibroblast pyridoxal kinase activities being identical in both ethnic groups (67). It has been suggested that the selective pressure of malaria has caused the reduced erythrocyte pyridoxal kinase activity (68). Recently an 8bp $PdxK$ promoter insertion with erythroid-specific properties has been reported (64). This insertion is less common in persons of African origin than in those of European or Asian ancestry and is thought to be the basis of a novel mechanism controlling the cell-specific activity of pyridoxal kinase (64).

The activity of pyridoxal kinase in rats responds rapidly and markedly to a dietary deficiency of $B_6$ becoming significantly lower than in those that are not $B_6$ deficient. The decrease in brain pyridoxal kinase activity however was not as marked as that seen in liver, muscle and plasma (38;69) indicating that pyridoxal kinase may play a role in the regulation of tissue levels of phosphorylated forms of vitamin $B_6$, protecting the PLP content of the brain during periods of $B_6$-deprivation (39).

1.1.7.3 Pyridox(am)ine 5'-phosphate oxidase (PNPO)

PNPO (E.C. 1.4.3.5) is a flavoprotein oxidase and catalyses the final step in the synthesis of PLP from pyridoxine and pyridoxamine i.e. the conversion of pyridoxine 5’-phosphate (PNP) and pyridoxamine 5’-phosphate (PMP) to PLP. This reaction also serves an important role in the salvage pathway whereby PLP is recycled in both $E.coli$ and higher organisms. Flavin mononucleotide (FMN) acts as a coenzyme for PNPO and is essential for catalytic activity (70;71).
The human form of PNPO has recently been cloned, expressed and the crystal structure investigated (70;72). The purified enzyme is a dimer composed of two identical subunits, each approximately 30 kDa with the two monomers interacting extensively along three regions of each subunit to constitute the functional dimer (70). The cofactor FMN is located in a deep cleft formed by the two subunits with extensive hydrogen-bond interactions to the protein (73).

The human PNPO gene maps to chromosome 17q21.32 and is composed of seven exons and six introns spanning over 7743 bp. The open reading frame encodes a protein of 261 amino acids (30;70). Analysis of the 5’-flanking region of the human PNPO gene sequence reveals that PNPO has several characteristics of a housekeeping gene including absence of a TATA-box, presence of Sp1-binding sites and the presence of CpG islands in the regulatory region (74).

In humans the tissue distribution of PNPO is widespread, consistent with its essential role in vitamin B₆ homeostasis. The specific activity of the enzyme varies depending on the tissue investigated however. The mRNA level of PNPO is highest in the liver; whilst skeletal muscle and kidney also contain considerable amounts of the transcript (34% and 86% relative to liver, respectively) lower levels are detected in the lungs (5%) (30). The level of mRNA in whole brain is surprisingly low (approximately 24%) compared to levels of expression seen in human adult liver.

### 1.1.8 Enzymes involved in catabolism of B₆ vitamers

#### 1.1.8.1 Aldehyde oxidase

Aldehyde oxidases are a small group of proteins belonging to the molybdo-flavoenzyme family (MFEs) which require FAD and molybdenum cofactor for their catalytic activity (75). All mammalian MFEs are homodimers consisting of two identical subunits which have a tripartite structure. Aldehyde oxidase is a member of the xanthine oxidase family and, unlike other molybdoprotein members, this family of enzymes requires the addition of a terminal sulphide ligand to the molybdenum centre to be catalytically active (76). This is catalysed by MoCo sulfurase (77).

The literature on aldehyde oxidases is limited. The human genome is characterised by a single functionally active aldehyde oxidase gene, \( Aoxl \), which maps to
chromosome 2q32.3-33.1. The most abundant source of AOX1 in man is in the liver (77;78) although individual levels of this enzyme in liver are known to be variable in the human population (77). Only one study has looked at the expression of AOX1 in the human central nervous system (78) where the presence of the Aox1 transcript was demonstrated in glial cells of the spinal cord. To date, no monogenic defects of mammalian aldehyde oxidase have been reported.

Little is known about the physiological role of aldehyde oxidases in mammals, although they are recognised to play an important role in the hepatic metabolism of drugs and potentially toxic compounds. Aldehyde oxidase(s) can act upon a variety of substrates which typically contain an aromatic heterocycle or an aromatic aldehyde. It is likely that aldehyde oxidase may play a role in the intermediary metabolism of many compounds and evidence suggests that pyridoxal may be one of its many physiological substrates (79;80).

1.1.8.2 NAD⁺-dependent aldehyde dehydrogenase

Studies involving rat mutants with a genetically determined absence of aldehyde oxidase suggest that an alternative pathway exists for the conversion of pyridoxal to 4-pyridoxic acid, via a NAD⁺-dependent aldehyde dehydrogenase (27). Animals lacking aldehyde oxidase activity excreted similar amounts of pyridoxic acid to those with high levels of aldehyde oxidase activity when challenged with a single dose of pyridoxal. Aldehyde dehydrogenase has been shown to be active in all tissues investigated, including brain, liver and red blood cells.

1.1.9 Vitamin B₆ deficiency states

The clinical effects of vitamin B₆ deficiency in infants were first described by Snyderman et al. (81;82). In the original study (hopefully never to be repeated) two infants were subjected to a diet containing 15% protein and completely devoid of vitamin B₆. Both infants showed a failure to gain weight; one developed convulsive seizures after 76 days and the second remained seizure free but showed a progressive hypochromic, microcytic anaemia. These clinical parameters were accompanied by biochemical changes consistent with vitamin B₆ deficiency and were corrected by the administration of pyridoxine.
Further knowledge of the clinical picture of vitamin B₆ deficiency emerged when 300 infants were inadvertently fed a diet deficient in vitamin B₆ (following the manufacture of a heat treated infant formula containing only 60 µg/L of vitamin B₆). The infants developed symptoms of central nervous system dysfunction described as a triad of hyperacusis, irritability and convulsive seizures. The symptoms developed between 6 weeks and 4 months of intake; they were aggravated by increased protein intake and diminished when infants were commenced on a carbohydrate diet (83). The clinical picture rapidly improved with an increased intake of dietary vitamin B₆.

Subsequent studies in healthy adult subjects on deficient diets have confirmed the following clinical signs and symptoms are associated with vitamin B₆ deficiency; eczema, seborrheic dermatitis, cheilosis, glossitis, angular stomatitis, microcytic and hypochromic anaemia, confusion, irritability, seizures and abnormal electroencephalograms (84;85)

1.1.10 Biochemical assessment of vitamin B₆ status

1.1.10.1 Indirect Methods

In the past several indirect measures to investigate vitamin B₆ status have been used. Alanine and aspartate transaminase (AST and ALT) both require PLP as a cofactor and activity of these enzymes in red cells (both with and without a saturating concentration of PLP) has been utilised as a measure of long term vitamin B₆ nutriture (86). Because this measure is functional rather than direct it is affected by factors other than PLP deficiency. Its use has also been limited by difficulties with defining normal reference ranges (87) and it is no longer useful as a tool of assessment.

Historically the tryptophan load has been the most widely used index of vitamin B₆ status. This test utilises the fact that the enzymes kynureninase and kynurenine aminotransferase in the metabolism of tryptophan require PLP as a cofactor. In vitamin B₆ deficiency the activity of these enzymes is reduced leading to increased formation and excretion of xanthurenic and kynurenine acids which can be measured in urine, particularly following an oral dose of tryptophan. Although this test remains a valid indicator of vitamin B₆ status, it is relatively invasive and time consuming and has been replaced by more sophisticated direct methods.
1.1.10.2 Direct Methods

PLP is the primary form of circulating vitamin B₆ in plasma and direct measurement of plasma PLP concentration is now considered one of the best indicators of vitamin B₆ status (88). It has been shown to correlate well with tissue (muscle) PLP in animal studies (69) and more recently simultaneous measurement of plasma and erythrocyte PLP using reverse phase HPLC methods demonstrated a strong positive correlation in healthy subjects. This did not hold true in a population of patients with a systemic inflammatory response however where PLP concentrations were reduced in plasma and elevated in red cells compared to controls (89).

Plasma PLP concentrations do respond to changes in vitamin B₆ intake although, unlike urinary 4-pyridoxic acid measurements, the change is not observed immediately. Studies have shown that plasma PLP levels plateau within 7-10 days of a change in vitamin B₆ intake and may take several weeks to return to pre-supplement levels on stopping increased intake (86;90). In contrast, urinary 4-pyridoxic acid excretion changes rapidly with a change in vitamin B₆ intake (86). Urinary 4-pyridoxic acid measurement may therefore be considered a good indicator of recent B₆ intake whereas plasma PLP is a good intake of the body store of vitamin B₆ (90).

1.1.11 Vitamin B₆ requirements

Estimates for vitamin B₆ requirements and Reference Nutrient Intakes (RNI) are based on depletion-repletion studies where either direct (plasma PLP concentration) or indirect (tryptophan load) measures have been used as a marker of vitamin adequacy (91-93).

As vitamin B₆ is central to overall protein metabolism it is likely that requirements are related to the amount of amino acid to be metabolised. In support of this Canham et al. (85) showed that vitamin B₆ depletion developed more rapidly in subjects on a high protein intake compared to those on low intakes.

Reference Nutrient Intake (RNI) for vitamin B₆ in adults in the UK is 15 µg/g dietary protein and for infants up to 6 months 8 µg/g dietary protein. The values are lower than for adults in part because some of the protein is required for tissue growth rather
than metabolised for energy (Dietary Reference Values for Food Energy and Nutrients for the UK, DOH 1991).

### 1.1.12 Variation of vitamin B₆ metabolism with age

PLP concentrations vary with age and research suggests that plasma and CSF PLP decreases from early childhood through to adulthood (94;95). Animal studies indicate that PLP is important in the developing foetus and during neonatal and infancy periods (96;97), however, our knowledge of B₆ metabolism and maturation of enzyme systems in this age group, particularly those of catabolism is incomplete. Improved understanding of these pathways is important for establishing normal reference ranges, when considering the nutritional requirements of neonates (including preterm infants), in the treatment of B₆ dependent disorders in neonates and management of inborn errors of B₆ metabolism during pregnancy.

A large-scale US population based study of >6000 participants aged over 1 year demonstrated that plasma PLP decreases with age after adolescence in men (94). Limited information is available from this study regarding concentrations in childhood and none about infancy. Gender differences in plasma PLP related to age are also described; the onset of reduced PLP at menarche with gradual increase following the menopause and a link to oral contraceptive pill use strongly suggests that oestrogen plays a role in determining plasma PLP (94). Bates et al. (98) studied vitamin B₆ status in the UK population and documented significantly lower plasma PLP in those >65years compared to the age group 4–18years. In addition 4-pyridoxic acid was significantly higher in the elderly group where it was strongly correlated to plasma creatinine as a marker of renal function. PLP concentration in CSF also shows an age related decrease (95;99) this is likely to be a reflection of plasma PLP however good studies measuring both simultaneously have not been conducted.

The possible causes of age related changes and low vitamin B₆ status in the elderly are multiple, however, a study in men of various ages suggested that B₆ absorption, phosphorylation (alkaline phosphatase levels) and excretion were not affected by age (100). Animal studies have provided a more detailed assessment of changes in B₆ metabolism with age. Rats show a decrease in plasma PLP with increasing age, the greatest decrease being in the first year of life (101). Functional tests of B₆
sufficiency (e.g. tryptophan load) are within normal limits however, indicating that despite lower concentrations, B₆ status is adequate in older animals. Alterations in vitamin B₆ tissue distribution are also observed; increasing concentrations of PLP and pyridoxamine phosphate in the brain and heart are described paralleled by a reduction in muscle PLP concentration with increasing age. The vitamin B₆-catabolising enzymes aldehyde oxidase and dehydrogenase show a significant increase in activity in the liver with a concomitant increase in urinary 4-pyridoxic acid excretion. Although significant differences between male and female rats are seen with respect to B₆ metabolism, trends with age are the same in both genders.

Evidence from human and animal studies suggests that vitamin B₆ sufficiency is very important during early development. B₆ adequacy is essential for normal development of the cerebellum, neocortex and caudate/putamen in rat pups (96;97) and in man structural brain abnormalities are seen in antiquitin deficiency. These observations may be secondary to the reduced activity of PLP dependent enzymes required for central nervous system development and neural migration for example, serine racemase (102). In addition, reports of families with pyridoxamine 5’-phosphate oxidase (PNPO) deficiency indicate a high rate of conception difficulties and early miscarriage further indicating a vital role for synthesis of PLP in early human development.

Studies of neonatal and infant vitamin B₆ metabolism indicate differences dependent on method of feeding which may relate to the form of vitamin B₆ present in milk. Human breast milk contains pyridoxal as the predominant form whereas artificial feeds contain only the heat stable form, pyridoxine (103). In healthy breast fed term infants, plasma PLP decreases strikingly over the first week of life and is significantly lower than plasma PLP in infants ingesting comparable volumes of formula milk (104;105). Significantly elevated plasma PLP concentrations (majority greater than 95th percentile) are consistently seen up to 9 months of age in formula fed compared to breast fed infants whose mothers take a pyridoxine supplement (106). Although no toxicity has been documented, various authors have questioned whether vitamin B₆ (pyridoxine) concentrations in formula preparations are too high, particularly in relation to protein content when compared to human breast milk [standard formula milk 40 μg PN/g protein compared to human milk 0.6 – 29 μg B₆/g
protein] (106). Similar concerns have also been raised regarding the B₆ (pyridoxine) content of parenteral feed preparations (107;108).

1.1.13 Inherited disorders of B₆ metabolism

Several inherited disorders of childhood have been described which result in an intracellular deficiency of the active cofactor via different mechanisms. Given the central importance of PLP in amino acid and neurotransmitter metabolism it is not surprising that these disorders present with a neurological phenotype frequently involving seizures. Affected infants may have characteristic amino acid and neurotransmitter amine metabolite profiles. Pathophysiological mechanisms in these conditions vary and are summarised in Tables 1 & 2 and are discussed in detail in the following sections 1.1.13.1 – 1.1.13.6.

Vitamin B₆, usually in the form of pyridoxine, may be used therapeutically in some heritable disorders where the primary defect is of a PLP-dependent enzyme. In this instance the principle of treatment is to augment any residual enzyme activity, such disorders are listed in Table 3.
Table 1. Disorders in which vitamin B₆ preparations (pyridoxine or pyridoxal 5’-phosphate) may be used therapeutically where metabolites accumulate that inactivate pyridoxal 5’-phosphate

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Enzyme</th>
<th>Chromosome, gene and location</th>
<th>Vitamin B₆ preparation and dose</th>
<th>Clinical aim or biochemical effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine dependent epilepsy</td>
<td>α-Aminoadipic semialdehyde dehydrogenase (Antiquitin)</td>
<td>EC 1.2.1.31</td>
<td>Pyridoxine 50-100 mg IV single dose(s) 5-15 mg/kg/d oral maintenance</td>
<td>Cessation and prevention of seizures and improvement of IQ</td>
</tr>
<tr>
<td>(266100 / 107323)</td>
<td></td>
<td>5q31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperprolinaemia type II</td>
<td>L-Δ¹-Pyrroline-5-carboxylic acid (P5C) dehydrogenase</td>
<td>EC 1.5.1.12</td>
<td>Pyridoxine 50 mg/d, subsequent reduction to 10 mg/d</td>
<td>Cessation and prevention of seizures (particularly during intercurrent infection)</td>
</tr>
<tr>
<td>(239510 / 606811)</td>
<td></td>
<td>1p36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EC – Enzyme Commission number
Table 2. Disorders in which vitamin B₆ preparations (pyridoxine or pyridoxal 5’-phosphate) may be used therapeutically where there is inadequate production of the active cofactor (in the correct location) due to an inborn error affecting B₆ interconversion

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Enzyme</th>
<th>Chromosome, gene and location</th>
<th>Vitamin B₆ preparation and dose</th>
<th>Clinical aim or biochemical effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal phosphate dependent epilepsy</td>
<td>Pyridox(am)ine 5’-phosphate oxidase (PNPO)</td>
<td>PNPO; 17q21.32</td>
<td>Pyridoxal phosphate 30-50 mg/kg/d orally</td>
<td>Cessation and prevention of seizures</td>
</tr>
<tr>
<td>(603287 / 610090)</td>
<td>EC 1.4.3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypophosphatasia (infantile)</td>
<td>Tissue nonspecific alkaline phosphatase (TNSALP)</td>
<td>ALPL / TNSALP / TNAP 1p36.1-34</td>
<td>Pyridoxine 100mg IV</td>
<td>Cessation and prevention of seizures</td>
</tr>
<tr>
<td>(241500 / 171760)</td>
<td>EC 3.1.3.1</td>
<td></td>
<td>Pyridoxal phosphate 30 mg/kg/d</td>
<td></td>
</tr>
<tr>
<td>Hyperphosphatasia</td>
<td>Phosphatidylinositol glycan class V (PIGV)</td>
<td>PIGV; 1p36.11</td>
<td>Pyridoxine 100 mg/day</td>
<td>Cessation of seizures, paradoxical change in electroencephalogram</td>
</tr>
<tr>
<td>(239300 / 610274)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EC – Enzyme Commission number
Table 3. Disorders in which vitamin B6 preparations (pyridoxine or pyridoxal 5’-phosphate) may be used therapeutically to augment residual enzyme activity in inborn errors affecting a PLP-dependent enzyme

<table>
<thead>
<tr>
<th>Disorder (OMIM reference)</th>
<th>Enzyme</th>
<th>Chromosome, gene and location (and mutations conferring B₆ responsiveness)</th>
<th>Vitamin B₆ preparation and dose</th>
<th>Clinical aim or biochemical effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocystinuria (236200)</td>
<td>Cystathionine β-synthase EC 4.2.1.22</td>
<td>CBS; 21q22.3 I278T, A114V, R266K, R336H, K384E, L539S</td>
<td>*Pyridoxine 100 mg/day</td>
<td>Normalisation of biochemical parameters. Prevention of thromboembolic events</td>
</tr>
<tr>
<td>(B6 responsive subgroup)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gyrate atrophy of the choroid and retina (258870)</td>
<td>Ornithine δ-aminotransferase EC 2.6.1.13</td>
<td>OAT; 10q26 V332M, A226V, E318K</td>
<td>Pyridoxine 500-1000 mg/day</td>
<td>Reduction of plasma ornithine. Unknown effect on chorioretinal degeneration</td>
</tr>
<tr>
<td>Aromatic amino acid decarboxylase deficiency (608643/107930)</td>
<td>Aromatic L-amino acid decarboxylase EC 4.1.1.28</td>
<td>AADC; 7p11</td>
<td>**Pyridoxine 400-800 mg/day ***Pyridoxal phosphate 200 mg/day</td>
<td>Improvement of Parkinsonian movement disorder</td>
</tr>
<tr>
<td>Disorder</td>
<td>Enzyme Family</td>
<td>Enzyme</td>
<td>Mutation</td>
<td>Dosage</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td>Pyridoxine responsive anaemia</td>
<td>$\Delta$-aminolevulinate $\delta$-ALA synthase</td>
<td>EC 2.3.1.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(X-linked sideroblastic anaemia)</td>
<td>Liver-specific alanine/glyoxylate aminotransferase</td>
<td>EC 2.6.1.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary hyperoxaluria Type I</td>
<td></td>
<td></td>
<td>c.508G&gt;A</td>
<td>Pyridoxine 5-10 mg/kg/day</td>
</tr>
<tr>
<td>(259900/604285)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(219500/607657)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathioninuria</td>
<td>$\gamma$-cystathionase</td>
<td>EC 4.4.1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoserine aminotransferase deficiency</td>
<td>Phosphoserine aminotransferase</td>
<td>EC 2.6.1.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(610936/610992)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McArdle’s disease; Glycogen Storage Disease Type V</td>
<td>Muscle glycogen phosphorylase</td>
<td>EC 2.4.1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(232600/608455)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Ensure patient is folate replete **Effective to augment L-dopa treatment (not as single therapeutic agent). Some cases report no improvement ***In twin patients being treated with tranylcypromine, pergolide and pyridoxine, substitution of pyridoxine with pyridoxal phosphate led to improved symptom control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EC – Enzyme Commission number
1.1.13.1 Antiquitin deficiency (pyridoxine dependent epilepsy, PDE)

Pyridoxine dependent seizures were first described by Hunt et al. in 1954 (109). In this first case report an infant with therapy-resistant seizures was found to respond dramatically to pyridoxine (in a multivitamin preparation) and remained seizure free on this treatment. A trial without pyridoxine resulted in seizure recurrence, hence the term ‘pyridoxine dependence’. For many years the underlying disease mechanism was not understood and many postulated that the PLP dependent enzyme, glutamic acid decarboxylase (GAD), was defective resulting in an imbalance between excitatory glutamate and inhibitory gamma aminobutyric acid (GABA). Following the observation that pipecolic acid is elevated in children with PDE (110), the underlying metabolic defect was finally shown to be a deficiency of α-aminoacidic semialdehyde (α-AASA) dehydrogenase, an enzyme lying on the catabolic pathway of lysine (111) (Figure 5). The accumulating upstream metabolite, L-Δ-piperideine-6-carboxylate (P6C) forms an adduct with PLP thus rendering it inactive as a cofactor. P6C is in equilibrium with α-AASA and it is measurement of these compounds in urine, CSF or plasma that now forms the biochemical basis for diagnosis, alongside molecular genetic analysis. Until recently elevated α-AASA was thought to be unique to individuals with PDE, however it is now also described in disorders of sulphite accumulation such as molybdenum cofactor and sulphite oxidase deficiency (112).
Following the availability of a diagnostic test for children with pyridoxine dependent epilepsy, our understanding of the phenotype and genotype of this condition has grown. In contrast to the initial, ‘classic’ description of this disorder of a neonatal epileptic encephalopathy which showed an ‘immediate and extraordinary’ response to treatment with pyridoxine (109), it is now recognised that not all children with PDE will show such a response in the early stages. This may be partly explained by the fact that many of these children suffer multi-system pathology for example, electrolyte disturbance, abdominal distension, feed intolerance and respiratory distress (113) which may contribute to seizure generation. Newborn infants with PDE are often in poor condition at delivery with evidence of fetal distress and poor Apgar scores and they may therefore be misdiagnosed as suffering hypoxic ischaemia encephalopathy (HIE). Conversely in some cases HIE may be considered a direct complication of PDE. A further interesting clinical feature noted in this population is the presence of structural brain abnormalities; this may also result in misdiagnosis of the seizure disorder and a missed treatment opportunity.
Additional clues to the diagnosis of a $\text{B}_6$ dependent seizure disorder may lie in the biochemical phenotype of plasma, urine or cerebrospinal fluid and is discussed below (Section 1.1.13.2).

PDE is an autosomal recessive disorder; to date more than 60 disease causing mutations have been published in the $\text{Antiquitin}$ gene with a common mutation in Exon 14 (E399Q) being found in approximately one third of patients. There is, as yet, no consensus as to whether a genotype-phenotype relationship exists in this disorder; Mills et al. (113) found no apparent relationship. However Scharer et al. (114) suggested that patients can be divided into three clinical phenotypes according to the degree of seizure response and developmental outcome and this phenotype can be related to the causative mutation, where milder phenotypes have some residual enzyme activity.

In the vast majority of patients, treatment with intravenous pyridoxine (100 mg single dose) followed by a maintenance oral dosing regimen of 5-10 mg/kg/day (maximum 200 mg per day) results in seizure resolution without the need for additional anticonvulsant medication. Seizure breakthrough during periods of intercurrent infection and fever is not uncommon (113;115).

The long term outcome in this condition is variable, although most children have a degree of developmental delay involving cognitive impairment and problems of speech and language. A few have normal cognitive development (115;116). Prospective treatment of an at-risk foetus by administration of pyridoxine to pregnant mothers or to newborn infants from birth has shown variable results where seizures are controlled but developmental problems remain (117;118). This suggests that there may be other pathogenic mechanisms at play in addition to the documented PLP deficiency. As the defect in PDE affects lysine catabolism, it has recently been proposed that the condition be considered as a cerebral organic aciduria. Within this paradigm accumulating toxic upstream metabolites may be amenable to additional treatment approaches such as dietary lysine restriction (115).
1.1.13.2 Pyridoxamine 5'-phosphate oxidase (PNPO) deficiency

PNPO deficiency is a recently described autosomal recessive disorder of vitamin B₆ metabolism. PLP-responsive, pyridoxine-resistant seizures were first reported in 2002 (119). A premature female infant with severe neonatal epileptic encephalopathy was described whose seizures, electro-encephalogram (EEG) and psychomotor development improved on PLP therapy where pyridoxine and conventional anticonvulsants had been unsuccessful. Further similar cases of pyridoxine resistant neonatal epileptic encephalopathy were reported subsequently (120;121) where biochemical investigations indicated deficiency of PLP dependent enzymes. PLP therapy was trialled to good effect in one case (122).

Mills et al. (123) first defined the molecular basis of PNPO deficiency in this group of patients (123) and to date 16 children from 8 families have been genetically confirmed or diagnosed by characteristic clinical phenotype in a union/family known to harbour mutations. In addition prenatal diagnosis has identified an affected foetus in two cases. These pregnancies were terminated.

Premature delivery (<37/40 weeks) is reported in the majority of genetically confirmed PNPO deficient cases reported in the literature. Patients are often in poor condition at delivery, many requiring intubation and PNPO deficiency should therefore enter the differential diagnosis of hypoxic-ischaemic encephalopathy in a prematurely born infant.

In the vast majority, seizures commence within the first hours of life and are frequently associated with a burst suppression pattern on electroencephalogram. Various seizure types have been described and in two cases seizure activity was of antenatal onset. Lactic acidosis has been described in several affected infants, the significance of which is unknown as it may simply be reflective of poorly controlled seizures in a sick neonate.
A difficult obstetric history should alert the clinician to a possible diagnosis of PNPO deficiency as heterozygous couples appear to have reduced rates of conception; many have undergone several attempts at in-vitro fertilisation treatment and suffered early pregnancy losses.

A clinical suspicion of antiquitin or PNPO deficiency is supported by biochemical analysis of plasma, urine and cerebrospinal fluid which may show evidence of impaired activity of the following PLP dependent enzymes; aromatic amino acid decarboxylase (reduced CSF HVA and 5-HIAA, elevated L-Dopa, 5-hydroxytryptohan and 3-methoxytyrosine; increased urinary vanillactic acid), threonine dehydratase (elevated CSF threonine), glycine cleavage enzyme (elevated CSF glycine) and ornithine δ-aminotransferase (reduced CSF arginine).

Measurement of CSF PLP and pyridoxal has shown reduced levels in a few reported cases of both PNPO deficient patients (95) and may prove to be important in other B6 related seizure disorders. As increasing numbers of genetically determined PNPO patients are reported it is apparent that some show non-characteristic metabolic profiles and in some instances the most characteristic findings may be present only transiently or absent altogether (124).

The long term outcome of PNPO deficient patients is unclear at present. Review of all cases published to date suggests that untreated, PNPO deficiency frequently leads to death from uncontrolled seizure activity. In the cases where treatment with PLP was initiated, the majority survive with varying degrees of neuro-developmental disability. It remains to be seen how prophylactic or early therapy with PLP may impact upon clinical outcome and, as with many rare diseases, more mild phenotypes may be identified over time.

The seizure disorder in PNPO deficient patients usually shows a good response to administration of PLP; burst suppression evident on EEG and biochemical parameters also resolve on treatment. Caution is required when PLP therapy is initiated however as two publications report complete EEG suppression (123;124) and in one case extreme hypotonia, apnoea and lack of responsiveness (.persisting for 4 days) before clinical improvement was seen (122;123).
Initial doses of 40-50 mg PLP have been administered via intravenous route (119) or by enteral route (123). Maintenance oral dosing regimes sufficient to suppress seizure activity are between 30 – 50 mg/kg/day with the majority of patients responding to 30 mg/kg/day 4-6 hourly (119;123-126).

Flavin mononucleotide (FMN) is known to be an essential cofactor for the PNPO enzyme and recent work using site-directed mutagenesis, enzyme kinetics and X-ray crystallography of the human protein suggests that patients with the particular mutation R229W may benefit from treatment with FMN (in the form of riboflavin, vitamin B₂) in addition to pyridoxal phosphate (127).

1.1.13.3 Other pyridoxal 5’-phosphate responsive patients

Although PNPO deficiency is considered a rare disorder, larger numbers of infants have been described in whom severe epilepsy is better controlled with the use of PLP than the use of pyridoxine (125). It is not yet known whether any of these infants have mutations or polymorphisms in the PNPO gene. PLP has also been used to successfully treat an adult patient with intractable status epilepticus (128).

In clinical practice, particularly in the Far East, pyridoxine and pyridoxal phosphate are commonly used as antiepileptic drugs in infantile spasms (129). In a case series of infantile spasms of various aetiologies, over 10% with known prenatal pathology of antenatal origin aetiology and over 20% with idiopathic spasms responded to pyridoxal phosphate (130). Another study by Wang et al. showed that PLP was effective in controlling up to 46% of patients with intractable infantile spasms. Patients with focal epilepsy and generalized epilepsy (excluding infantile spasms) had a PLP response rate of 8% and 5%, respectively. A single case of paradoxical increase of seizure activity after PLP treatment is also reported highlighting that caution is required in PLP therapy (131).

Maintenance of optimal PLP levels in the brain is likely to be important in many neurological disorders where neurotransmitter metabolism is disturbed either as a primary or as a secondary phenomenon.
1.1.13.4 Hypophosphatasia

This inherited disorder is due to deficient activity of tissue-nonspecific alkaline phosphatase, the biochemical basis of which is discussed in detail above (Section 1.1.7.1). Clinically it is divided into six classes; only the lethal perinatal and infantile forms show an autosomal recessive pattern of inheritance (132). All affected individuals have defective skeletal mineralisation, however, disease expression is remarkably variable. Infants affected by the perinatal form may be affected in utero and are stillborn, whereas at the other extreme some individuals present in adulthood with hypophosphatasia characterised by osteomalacia and recurrent metatarsal stress fractures. All forms show extracellular accumulation of the endogenous products, phosphoethanolamine (PEA), inorganic pyrophosphate (PP\(_i\)) and PLP. The level of PLP accumulation is the most sensitive and specific substrate marker for hypophosphatasia and appears to be proportional to disease severity (132). Although elevated PLP levels per se are not thought to be disease causing, the elevation in extracellular PLP is not reflected within the cell where low levels of PLP are likely implicated in the generation of seizures seen in some patients with infantile hypophosphatasia (see above). Several cases of successful seizure treatment with vitamin B\(_6\) have been reported (52;133;134).

1.1.13.5 Hyperphosphatasia

Hyperphosphatasia mental retardation or Mabry syndrome was initially described in a single family and characterised by severe developmental delay, seizures and greatly elevated serum levels of tissue non-specific alkaline phosphatase (135). A subsequent case report also documents a significantly reduced plasma PLP (6 nmol/L) in one affected patient in whom seizure activity and alertness showed improvement with pyridoxine treatment (136).

The molecular basis of a group of patients with this clinical phenotype ‘Mabry syndrome’ has recently been defined by ‘identity-by-descent’ filtering of exome sequence data (137). Mutations were identified in the \(PIGV\) (phosphatidylinositol glycan anchor biosynthesis class V) gene of four families with the characteristic clinical phenotype. \(PIGV\) is the second mannosyltransferase in the glycosylphosphatidylinositol (GPI) anchor biosynthesis pathway which is required
to anchor tissue nonspecific alkaline phosphatase to the cell membrane where it is functional. The seizures observed in this condition may therefore theoretically relate to a reduced concentration of intracellular PLP. Many patients who have the same clinical picture do not harbour mutations in this gene thus it is likely that other genes critical to GPI anchor biosynthesis are likely to be disrupted in some patients (138).

1.1.13.6 Hyperprolinaemia type II

This rare inborn error of metabolism illustrates a mechanism of vitamin B₆ deficiency similar to that seen in Antiquitin deficiency. The disorder results from a deficiency of Δ1-pyrolase 5-carboxylate dehydrogenase and is characterised by elevated plasma proline and increased urinary excretion of proline, hydroxyproline and glycine. It is the accumulation of pyrroline 5-carboxylate (P5C) which adducts with PLP that is thought to lead to vitamin B₆ deficiency (139) (Figure 6). Clinically the condition is characterised by seizures which, in at least one reported case, show response to pyridoxine (140).
1.1.14 Drugs and Vitamin B₆

It is long known that vitamin B₆ may interact with several therapeutic drugs leading to hypovitaminosis and associated clinical symptoms. This may occur by one of two mechanisms, or a combination of both. Firstly via the interaction of the reactive aldehyde group of PLP with amine or hydrazine groups forming a Schiff base and rendering PLP inactive as a cofactor or secondly via inhibition of enzymes involved in B₆ vitamer metabolism, for example pyridoxal kinase (1;141).
1.1.14.1 Isoniazid (Isonicotinic acid hydrazide)

Isoniazid reacts non-enzymatically with PLP forming an inactive hydrazone complex (142) which may lead to a functional deficiency of PLP (Figure 7). Evidence of deranged vitamin B₆ metabolism is observed in patients receiving antituberculous therapy with isoniazid. Increased urinary excretion of xanthurenic acid following a tryptophan load which normalises with pyridoxine (143;144) and secondary pellagra due to impaired activity of PLP dependent kynureninase are both described (144).

The ability of isoniazid to cause severe clinical vitamin B₆ deficiency is also evident in isoniazid overdose. Multiple case reports and short series have illustrated pyridoxine as a successful antidote in acute overdose and in long term chronic over-ingestion (144-146).

![Figure 7. Isoniazid interaction with pyridoxal 5'-phosphate (PLP)](image)

1.1.14.2 L-Dopa

PLP forms a Schiff base complex (tetrahydroisoquinoline compound) with L-dopa rendering both L-dopa and PLP functionally inactive (147). When pyridoxine was given to Parkinsonian patients to control nausea associated with L-dopa therapy a considerable reduction in its efficacy was noted (148). Following these observations, historically vitamin B₆ supplementation was contraindicated during treatment with L-dopa however current practice of including a peripheral decarboxylase inhibitor prevents this complication.
More recently Miller at al. (149) showed that elevated plasma homocysteine in Parkinson disease subjects on treatment with L-dopa correlated inversely with serum folate, B\textsubscript{12} and PLP concentrations compared to normal controls and drug naïve Parkinsonian patients. Plasma PLP concentrations in the L-dopa treated group were significantly lower than in the control group.

B\textsubscript{6} supplementation during L-dopa treatment (with a decarboxylase inhibitor) in Parkinson’s disease may be beneficial in reducing plasma homocysteine levels thus limiting its potential adverse effects on the cardiovascular system. There may be other benefits of concurrent administration of pyridoxine in Parkinsonism. The effect of loss of dopaminergic neurons may be further compounded by a secondary AADC deficiency resulting from depletion of its cofactor PLP. Pyridoxine administration could correct this and thereby boost both endogenous dopamine synthesis and dopamine synthesis from the medicinal L-Dopa.

1.1.14.3 Methylxanthines including theophylline

Theophyllines used in the treatment of respiratory disorders are potent competitive inhibitors of pyridoxal kinase (150;151). In keeping with this, asthmatic patients have been shown to have significantly lower plasma and erythrocyte PLP compared to normal controls although no relationship to medication was made during this study (152). Theophylline-induced seizures which may be encountered during overdose have significant morbidity and mortality and, although pyridoxine treatment is shown to be beneficial in animals, in practice these seizures remain very difficult to treat (153;154).

1.1.14.4 Anticonvulsants

Abnormalities of vitamin B\textsubscript{6} metabolism in children taking antiepileptic medications were first observed by Reinken (155). He postulated that the ‘depression’ in erythrocyte glutamic oxaloacetic transaminase (EGOT, a PLP-dependent enzyme) and PLP observed on treatment with antiepileptic drugs (AED) was secondary to inhibition of pyridoxal kinase and that administration of vitamin B\textsubscript{6} should be considered in conjunction with AED. Subsequently several studies have demonstrated a reduction in plasma PLP following the use of various anticonvulsants. Much of this work has been stimulated by the concurrent and likely
related observation of hyperhomocystinaemia which may be associated with long term cardiovascular risk. The importance of plasma PLP levels in these children is not only limited to homocysteine metabolism; experimental evidence and clinical experience indicates that a steady state concentration of PLP is essential for electrochemical activity of the brain, thus treatment of low PLP levels in epileptic children on AED may warrant consideration in cases of poor seizure control.

The mechanism of PLP reduction observed with AED treatment in the majority of studies is not fully understood. Several AED (phenytoin, phenobarbitone, carbamazepine and primidone) are potent hepatic inducers of cytochrome P450 and other enzyme systems. For these medications, it is possible that induction of enzymes involved in the catabolism of PLP lead to reduced plasma PLP values. Phenobarbitone for example is known to induce aldehyde dehydrogenase (ALDH2) in the mouse (156) and ALDH2 is important for the detoxification of highly reactive aldehyde molecules such as pyridoxal. The finding of low urinary pyridoxic acid in epileptic patients on AED (157;158), however, is not consistent with increased PLP catabolism and is more suggestive of pyridoxal kinase or PNPO inhibition as originally hypothesised by Reinken (155). Many new AED are not hepatic enzyme inducers and little evidence exists regarding their effect upon PLP in long term treatment.

1.1.15 Pyridoxine and PLP toxicity

1.1.15.1 Acute toxicity of pyridoxine

In animal studies very high doses of pyridoxine (2-6 gram/kg) induce ataxia, seizures and may be lethal (159). Administration of such high doses is not reported in human studies, however pyridoxine appears to have low acute toxicity in man with doses of up to 357 mg/kg used for a short time period in isoniazid overdose without any adverse events (160).

1.1.15.2 Chronic toxicity of pyridoxine

Studies of chronic, long-term pyridoxine administration in animals have shown the potential neurotoxicity of vitamin B₆ with doses of 200 mg/kg causing ataxia, peripheral neuropathy and muscle weakness. Histological examination demonstrates
widespread neuronal damage with loss of myelin and degeneration of sensory fibres in peripheral nerves, dorsal columns of the spinal cord and descending tract of the trigeminal nerve (161). Neurotoxicity of injected pyridoxine may be enhanced by a protein deficient diet. This is perhaps due to decreased protein binding in serum and decreased urinary excretion of the toxin secondary to oliguria which is evident in these animals because of reduced water consumption (162).

Clinical and electrophysiological evidence of sensory neuropathy is observed in healthy volunteers and patients taking pyridoxine for a variety of indications. A single case reports evidence of sensory and motor neuropathy following a prolonged course of relatively high intake (1 gram/day for 10 years) (163). In the majority of cases, symptoms are reversible on stopping pyridoxine intake (although residual nerve damage remains in some patients) (164) and show a clear relationship to pyridoxine dose and in some instances to length of administration (93;165).

Vitamin B₆ (pyridoxine and pyridoxal phosphate) may paradoxically be pro-convulsant; in young rats epileptiform EEG changes and frank seizures were induced by intraperitoneal pyridoxine (166) and Hammen et al. (131) report a newborn with intractable epilepsy who showed an increase in seizure frequency and EEG alterations after administration of vitamin B₆. Wang et al. (167) also describe a paradoxical seizure increase during treatment with PLP. Recently a case of presumed pyridoxine toxicity resulting in status epilepticus was reported in a newborn at-risk of PDE who was treated with pyridoxine pending α-AASA results. Antiquitin deficiency was excluded and seizures resolved with pyridoxine withdrawal (168).

The mechanism of nerve damage observed in vitamin B₆ supplementation is unknown. Pyridoxal and 4-pyridoxic acid rise a disproportional amount compared to PLP (which may show little elevation) following supplementation and these vitamers may be responsible for toxicity. Supporting this, pyridoxal is known to be cytotoxic to cells including Schwann cells in culture (93).
1.1.15.3 Toxicity of pyridoxal 5'-phosphate

Less is known about PLP toxicity as clinical experience is comparatively limited. Hammen et al. (131) report a newborn with intractable epilepsy who showed an increase in seizure frequency and EEG alterations after administration of vitamin B₆. PLP may also cause tonic-clonic convulsions in immature mice possibly secondary to impaired GABA-ergic neurotransmission (169). PLP at a dose of 1800 mg/day induced hepatotoxicity in a child with homocystinuria (170) and liver cirrhosis has developed in one child with PNPO deficiency while on treatment with oral PLP (unpublished observation). The mechanism of toxicity is unknown, however, preparation of PLP immediately prior to administration may prevent deterioration of the vitamin in UV light on standing.

1.2 SEROTONIN METABOLISM AND FUNCTION

Serotonin is an indolealkylamine compound that is distributed widely throughout the animal and plant kingdom. It was first identified in 1948 (171) by a group researching vasoconstrictors that cause hypertension and was named for its presence in serum (sero) and its vasoactive abilities (tonin). Since this time a huge amount of research has focussed upon this important compound which is now known to play many diverse roles in human physiology and thus impact upon many disease processes, including several neuropsychiatric diseases.

1.2.1 Biosynthetic pathway of serotonin

Serotonin is synthesised by a two-step pathway from the essential amino acid tryptophan which is ingested in the diet (Figure 8). Access of tryptophan to serotonin-producing cells is a critical step in the regulation of serotonin biosynthesis and is discussed in later a chapter (Section 6.5.1). In the first rate-limiting step, L-tryptophan is hydroxylated to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH). Unlike serotonin, 5-hydroxytryptophan is able to cross the blood brain barrier (172), although the precise mechanism by which it does so is unknown. In the second step, which is catalysed by PLP dependent L-aromatic
aminoacid decarboxylase (AADC), 5-hydroxytryptophan is decarboxylated to serotonin.

![Figure 8. Synthetic pathway of serotonin and breakdown to 5-hydroxyindolacetic acid (5-HIAA)](image)

(i) tryptophan hydroxylase (ii) aromatic amino acid decarboxylase

**1.2.1.1 Tryptophan hydroxylase 1 and 2 (TPH1 and TPH2)**

Until recently it was assumed that TPH found in the periphery and central nervous system was derived from the same, single gene located on chromosome 11 (173). A second TPH isoform (TPH2) was described in 2003, however, that derived from a different gene lying on the long arm of chromosome 12 and was highly expressed in brain (174;175). The discovery of this second isoform was particularly interesting because this region of chromosome 12 had been reported in several studies to contain susceptibility genes for depression and bipolar disorder (176;177).

TPH1 contains 444 amino acids corresponding to a molecular weight of 51kDa and TPH2 contains 490 amino acids with a molecular weight of 56 kDa. Both isoforms catalyse the hydroxylation of tryptophan to 5-hydroxytryptophan and utilise molecular oxygen and tetrahydrobiopterin as a cofactor. They are tetrameric holoenzymes and are not fully saturated in normal physiological situations so it follows that increased substrate in the form of tryptophan is able to increase serotonin production.

TPH has a relatively limited pattern of expression; TPH2 is detected exclusively in the brain (174) raphe nuclei and pineal gland (178), while TPH1 is found in enterochromaffin cells of the gut (duodenum) (179), mast cells and lung (173).
The catalytic activity of TPH can be altered by several factors. It is regulated by Ca\textsuperscript{2+} and action-potential dependent phosphorylation and also by a specific activating protein (named 14-3-3) which can bind to the enzyme in its phosphorylated form to prevent deactivation by dephosphorylation (180). The activity of TPH is also modified by stress which is shown to increase activity and hence production of serotonin in the forebrain and midbrain raphe nuclei (181). Much of this work was completed before the second TPH isoform was identified therefore it is not known whether these features apply to both or only one specific isoform.

### 1.2.1.2 Aromatic L-amino acid decarboxylase (AADC)

AADC was first identified in 1938 from mammalian kidney extracts (182). AADC protein is a homodimer consisting of two monomers, each of 480 amino acids (183). Each homodimer binds two molecules of pyridoxal phosphate at lysine residue 303 within the active site, forming an internal aldimine through a Schiff base linkage, as is the case for many PLP dependent enzymes (184). Despite conjecture over many years, it is now accepted that AADC is a single enzyme that can catalyse the decarboxylation of several different substrates (185). The most important of these are 5-HTP to form serotonin and L-DOPA to form dopamine, however it is also considered that AADC is the sole enzyme responsible for the formation of the trace amines 2- phenylethylamine, p- tyramine and tryptamine, which may play a role in the modulation of central neurotransmission.

The anatomical distribution of AADC is wide; it is expressed in the central and sympathetic nervous systems, as well as in adrenal chromaffin cells. AADC is also present in D-neurons (non-monoaminergic cells) and throughout a number of non-neuronal tissues, many of which belong to the amine precursor uptake and decarboxylation (APUD) system. These tissues include kidney, liver, pancreas, gastrointestinal tract and lungs (186).

In man the AADC protein is encoded by a single gene on chromosome 7p21.1 – p12.3. It is over 85 kb in length and comprises 15 exons. Several splice variants have been identified in human and animal tissues which show differences in the coding and non-coding regions which likely direct tissue specific expression.
1.2.2 Synthesis of melatonin from serotonin

Serotonin not only has many important biological effects of its own, but it is also the precursor of melatonin. This hormone is essential in human biology for regulation of the normal sleep-wake cycle and also plays a role in many processes including sexual maturation and reproductive behaviour, thermoregulation, immune responses and reduction of oxidative stress (173;187).

Melatonin is synthesised from serotonin in the pineal gland which contains all the enzymes necessary to produce it from the precursor tryptophan. A unique feature of pineal physiology is that melatonin synthesis is significantly influenced by the dark-light cycle. The rate-limiting enzyme, serotonin N-acetyl transferase, converts serotonin to N-acetylserotonin and this enzyme displays significant circadian rhythm, with peak activity during darkness. The product is then methylated to form melatonin by the enzyme 5-hydroxyindole-O-methyltransferase which uses S-adenosyl methionine as the methyl donor (188) and does not show circadian variation. Thus serotonin N-acetyl transferase activity regulates the circadian rhythm of melatonin synthesis.

1.2.3 Metabolism, storage and mechanisms of action of serotonin

Serotonin is a hydrophilic molecule that does not readily pass the blood brain barrier thus serotonin present in the central nervous system must be synthesised within the brain. Serotonin-containing neuronal cell bodies are restricted to discrete groups which are located in the midline of the brainstem, primarily within the ‘raphe nuclei’ (189). The axons of this small group of neuronal cell bodies project widely to innervate nearly every region of the central nervous system and contain both TPH and AADC which are necessary for serotonin synthesis (188;190).

Serotonin is stored in vesicles within the serotonergic neurone and this requires its active transport from the cytoplasm. The vesicular monoamine transporter (VMAT) utilises the electrochemical gradient generated by an H⁺-ATPase to drive transport, where efflux of H⁺ is coupled to uptake of serotonin. Serotonin containing vesicles differ from those storing catecholamines as they contain virtually no ATP. They also show differences to the serotonin vesicles in enterochromaffin cells because they
contain a specific protein (serotonin-binding protein, SBP) that binds serotonin with high affinity in the presence of Fe$^{2+}$ (191).

Evidence derived primarily from cultured serotonergic synapses of the leech, suggests that serotonin is released from neurones through the process of exocytosis (192). Serotonin is released in response to depolarisation and is dependent on the presence of external calcium, the presynaptic resting potential and the magnitude of the depolarisation (192).

Serotonin has its effect in the central nervous system through interaction with numerous post-synaptic receptors. As with many other neurotransmitters, its actions are terminated within the synaptic cleft by binding to a specific transporter, the serotonin reuptake transporter (SERT). Via this mechanism serotonin is taken back up into serotonergic neurones where it may be repackaged into a vesicle or catabolised to 5-HIAA as detailed below (Section 1.2.4). The activity of SERT plays an important physiological role as it regulates the amount of serotonin within the synapse, thereby influencing the rate of synaptic transmission. This explains why drugs which block this transporter, such as selective serotonin reuptake inhibitors (SSRI’s), are effective in treating depression and obsessive compulsive disorder (193).

In man SERT is encoded by SLC6A4 which maps to chromosome 17q11.2. It is composed of 15 exons spanning 40kb and the predicted protein of 630 amino acids has 12 transmembrane domains (194). SLC6A4 is known to have alternative promoters and to contain many single base variations that are likely to regulate gene expression. Amongst others, the well-studied 5HTTLPR (serotonin-transporter-linked polymorphic region) promoter region variant and two single nucleotide polymorphisms (SNPs) [rs25531 and rs25532] located upstream of the transcription start site are known to effect the transcriptional rate of this gene. Several different SNPs are known to be associated with neuropsychiatric disorders including obsessive-compulsive disorder and autism (194).

Serotonin has three different modes of action; as a neurotransmitter it acts locally at synaptic boutons; it can act in a paracrine fashion upon diffusion at a site distance from its release and by circulating in the blood it produces effects as a hormone
At the majority of sites the function of serotonin is mediated through its interaction with the serotonin receptor. There are seven classes of serotonin receptor, six of which are G-protein linked and one which is a ligand gated ion channel. The G-protein linked receptors are further subdivided into 13 subtypes. These receptors are present both within the central and peripheral nervous system and although all can be activated by serotonin, differences in signal transduction mechanisms, anatomical distribution and differential response to pharmaceutical agents allow them to be distinguished. Understanding of this important group of receptors has increased greatly over the past decade with advances in cell, molecular and structural biology and serotonin receptors are the target for a large number of pharmaceutical drugs, many of which are used worldwide to treat common psychiatric disorders such as depression, anxiety and schizophrenia.

Despite its important role as a neurotransmitter in the central nervous system, the vast majority (approximately 95%) of serotonin is produced and stored in the enterochromaffin cells of the gut. These cells are a subtype of enteroendocrine cells and are found among the enterocytes of the intestinal epithelium. Enterochromaffin cells are able to synthesise serotonin from dietary tryptophan as they contain both tryptophan hydroxylase (TPH1) and aromatic aminoacid decarboxylase enzymes. Newly produced serotonin is then packaged into vesicles by a specific isoform of the vesicular monoamine transporter 1 (VMAT1) which is unique to enterochromaffin and adrenal chromaffin cells. Release of serotonin from vesicles on the basal border of the enterochromaffin cell is triggered by mechanical stimuli, pH and various nutrients including fatty acids, peptides and glucose. On release it appears to act in concert with other mediators (cholecystokinin and ATP) to modulate a large number of complex gastrointestinal reflexes which are specific to the content and region of the intestine. Serotonin may interact with immune cells and nerve terminals to exert its effects in the gastrointestinal system or it may be taken up into platelets.

As described previously, the actions of serotonin are terminated by its reuptake via the serotonin reuptake transporter (SERT) which is expressed in the gut on the apical and basal membranes of the epithelial cell. Here it is degraded to 5-
hydroxyindolacetic acid (5-HIAA) by the action of monoamine oxidase A and excreted.

Serotonin that is not metabolised in this manner may enter the portal circulation where it is taken up into the platelets via the same transporter, SERT. Serotonin in the platelets is protected from breakdown in the liver and enters the general circulation. Any remaining free serotonin in the portal circulation is rapidly catabolised by liver enzymes to 5-HIAA for excretion (197).

1.2.4 Catabolism of serotonin

As serotonin is a biologically potent molecule, tight regulation is important and this is achieved, at least in part, through catabolic processes. The main catabolic pathway of serotonin is oxidative deamination by the enzyme monoamine oxidase (MAO). MAO (which exists as type A and type B; see below) converts serotonin to 5-hydroxy-indoleacetaldehyde which is subsequently oxidised by NAD⁺ dependent aldehyde oxidase to form 5-hydroxyindolacetic acid (5-HIAA) (Figure 9) (188). 5-HIAA can pass the blood-brain-barrier and is secreted from the liver into the circulation for excretion in the urine. An alternative, minor pathway of serotonin catabolism of unknown significance involves reduction of 5-hydroxy-indoleacetaldehyde by aldehyde reductase to 5-hydroxytryptophol.

MAO exists in at least two forms; MAO type A and MAO type B, and both are integral flavo-proteins of the outer mitochondrial membrane. Evidence suggests that serotonergic neurones contain predominantly MAO type B, the form that does not preferentially degrade serotonin which has led researchers to suggest that MAO in the serotonergic neurone primarily acts to prevent accumulation of other substrates such as dopamine that could affect serotonin storage and function (188).

Recently a new serotonin metabolite (4R)-2-[(5’-hydroxy-1’H-indol-3’-yl) methyl] thiazolidine-4-carboxylic acid (5’-HITCA) has been identified which is thought to be formed as a result of cyclization between 5-hydroxy-indoleacetaldehyde and L-cysteine (202). The biological relevance of this is, at present unknown.
1.2.5 Physiological functions of serotonin

In man serotonin has a wide range of diverse physiological functions; its receptors being almost ubiquitously expressed (203). In the periphery, serotonin plays an important role in regulation of the smooth muscle both in the gastrointestinal and cardiovascular systems and it is also intimately involved in platelet aggregation. In the central nervous system, the actions of serotonin as a neurotransmitter impact upon behaviour, cognition and learning. The most important functions are summarised in the following sections 1.2.5.1 – 1.2.5.5.
1.2.5.1 Neurological and behavioural

All regions of the brain express serotonin receptors in a regional subtype-specific fashion (204) hence serotonin modulates virtually all human behaviours to some extent. Perhaps surprisingly, serotonergic neurones are present in only a limited area of the central nervous system, in the brainstem raphe nuclei, however they project widely to cortical, limbic, midbrain and hindbrain structures.

Over many decades, serotonin has been implicated in a large and diverse number of neuropsychological and behavioural processes. These include the modulation of mood, perception, anger, aggression, sexuality, reward and appetite (193). Consequently it has been proposed that serotonin plays a role in many and varied neuropsychiatric conditions in man including depression, anxiety and eating disorders. Definitive evidence of the mechanism by which serotonin is involved in such conditions is lacking, nevertheless treatment with drugs acting upon serotonin metabolism, receptors and transporters is effective in many cases.

There is no simple relationship between the brain region in which a specific receptor is expressed and the effect of serotonin upon a particular subtype of receptor as it appears that each human behaviour may be regulated by multiple serotonin receptors and in addition each subtype of receptor may be expressed in multiple brain regions (204). This observation, which is drawn primarily from behavioural studies in animals, explains why drugs targeted to a specific serotonin receptor have effects on several elements of behaviour (205) and reflects the complexity of human psychology.

1.2.5.2 Gastro-intestinal tract

Over 90% of serotonin in the periphery is contained within enterochromaffin cells of the gut (206). Within the gastro-intestinal tract serotonin is released in response to various stimuli including acetylcholine, raised intraluminal pressure and low pH (207) and it acts in a paracrine fashion as a signalling molecule to activate neural reflexes. It is thought to play an important role in intestinal secretion, peristalsis and the sensation of discomfort, nausea and in the gastro-intestinal tract. This area of physiology is at present incompletely understood and the relationship of serotonin to
gastrointestinal pathologies such as inflammatory bowel disease and irritable bowel syndrome is an area of intense research activity (203).

1.2.5.3 Cardiovascular system

Animal studies suggest that serotonin is important for normal cardiac development; tryptophan hydroxylase I knockout mice may develop fatal dilated cardiomyopathy (208) and the serotonin receptor subtype 2B (5-HT2B) receptor knockout mice have morphological cardiac abnormalities which lead to fetal and neonatal death (209). It is also thought that serotonin may play a pathological role in development of the cardiac valvulopathy associated with the weight loss medication fenfluramine (which has now been withdrawn) and the fibrosis of valves seen in carcinoid syndrome (see Section 1.2.7) (204;210).

Via its central neurotransmitter effects serotonin can influence cardiovascular status by its actions on the neurones of the raphe nuclei. Serotonin may affect both sympathetic and parasympathetic pathways thus having both chronotropic and inotropic effects on the cardiovascular system mediated by the 5-HT1, 5-HT2 and 5-HT3 serotonin receptors (208;211).

Serotonin is also involved in various elements of vascular biology including the modulation of vascular resistance and blood pressure, and the control of haemostasis (204). Serotonin is versatile in its effect on blood vessels, having differing effects according to the vascular bed, and thereby type of receptor, present (212).

Platelets contain a large amount of serotonin within ‘platelet dense granules’ in the cytoplasm (213). They do not synthesise serotonin directly but take it up from the plasma via the serotonin reuptake transporter (SERT). During activation following endothelium damage or ischaemia for example, serotonin is released from the platelet. It then plays a role in haemostasis by promoting aggregation of platelets via the serotonin receptor (5-HT2A) and causes vasoconstriction of surrounding blood vessels by a direct effect (203). This is borne out by the observation that tryptophan hydroxylase I knockout mice (which exhibit markedly decreased peripheral serotonin) have a decreased thrombotic and thromboembolic risk and impaired haemostasis (175).
1.2.5.4 Genito-urinary

Serotonin acts both centrally and in the periphery to modulate micturition and ejaculatory function. Through its action on 5-HT2C and 5-HT1B receptors, serotonin increases ejaculatory latency and delays orgasm (214) which has important implications for treatment of sexual dysfunction. It has opposing actions upon micturition according to the receptors upon which it acts; 5-HT2C receptors prevent urination whereas 5-HT1A receptors promote urination (215).

1.2.5.5 Glucose homeostasis

Serotonin is known to have an effect upon glucose metabolism however research to date is inconclusive as to its precise role. While some studies suggest that serotonin may increase blood glucose (216) more evidence suggests that it can lead to hypoglycaemia. This observation may be mediated by the 5-HT2A receptor which is expressed in skeletal muscle as serotonin action at this receptor causes rapid glucose uptake (217;218). Insulin does not appear to be involved in the serotonin mechanism of hypoglycaemia as tryptophan administration to rats does not result in increased insulin levels (219;220).

1.2.6 Inherited disorders associated with an abnormality of serotonin metabolism

Several rare inherited diseases lead to a central serotonin deficiency, usually in association with reduced amounts of the other monoamine neurotransmitters, dopamine and norepinephrine (221). A monogenic disorder resulting in isolated deficiency of tryptophan hydroxylase I or II has yet to be described however, this enzyme requires tetrahydrobiopterin (BH₄) as an essential cofactor, and a secondary deficiency is encountered in clinical cases of sepiapterin reductase, 6-pyruvyltetrahydrobiopterin synthase (PTPS) and GTP cyclohydrolase (GTPCH) deficiency which affect BH₄ synthesis and in dihydropteridine reductase (DHPR) deficiency which affects BH₄ recycling. None of these disorders produce isolated serotonin deficiency however because tyrosine hydroxylase utilises the same cofactor hence production of dopamine is also severely affected. Clinically these conditions are characterised by a hypokinetic-rigid syndrome often accompanied by
oculogyric crises, ptosis and autonomic dysfunction and it is very difficult to dissect out what symptoms are attributable to serotonin deficiency alone. One detailed case study of an adult with sepiapterin reductase deficiency demonstrated restoration of a severely disturbed circadian sleep-wake cycle accompanied by normalisation of CSF 5-HIAA and melatonin profile, in response to treatment with 5-HTP but not levodopa (222). This study is indicative of the central role that melatonin plays in maintenance of circadian rhythm and the authors highlighted that non-motor aspects of these disorders are often poorly recognised.

In addition to the disorders discussed above, deficiency of aromatic amino acid decarboxylase (AADC) or its cofactor PLP (for example in PNPO or Antiquitin deficiency) may result in a combined deficiency of both serotonin and dopamine which is evidenced by reduced HVA and 5-HIAA in the cerebrospinal fluid. Although classically patients with AADC deficiency present with a similar clinical picture to that described above, disorders of vitamin B₆ metabolism tend to be dominated by a seizure disorder with few reported cases of movement disorder or of symptoms obviously attributable to serotonin deficiency. In keeping, not all patients have a demonstrable abnormality in the neurotransmitter profiles and pathology in these cases is likely related to one of the many diverse cofactor roles of vitamin B₆.

Several clinical cases without a definitive diagnosis have been reported in the literature where there is evidence of reduced serotonin or its metabolites, accompanied by a variety of clinical symptoms. In one such child a syndrome of hypotonia, developmental delay, ataxia and atypical autism with isolated low CSF 5-HIAA was described (223). A heterozygous gain-of-function change was found in the SLC6A4 gene encoding SERT in addition to a homozygous 5-HTTLPR L/L promoter variant and the authors postulate that impaired serotonergic neurotransmission may be due to increased SERT activity (223). Treatment with 5-hydroxytryptophan was reported to normalise the biochemical findings and improve clinical symptoms.

A further group of five patients have been reported who also show amelioration of clinical symptoms (hypotonic-ataxia and poor attention) and biochemical serotonin deficiency with 5-hydroxytryptophan and carbidopa combination therapy (224). In this report the authors postulate a regulatory abnormality in the tryptophan
hydroxylase gene to be disease causing as no deleterious changes were demonstrated in the coding regions.

A clinically different case is reported by Lin et al. (225) that shares some of the biochemical features of serotonin deficiency discussed above. This child presented in the neonatal period with seizures and transient non-ketotic hyperglycaemia evident in the CSF. He initially responded to anticonvulsant medication (but not intravenous pyridoxine) but represented with status epilepticus at 2 months of age. At this time CSF glycine had normalised however a severely reduced, isolated CSF 5-HIAA level and platelet serotonin level was observed. Multiple treatment approaches were trialled with variable success, including 5-hydroxytryptophan and carbidopa however he progressed to have a cognitive and motor developmental delay as well as abnormalities of social behaviour and communication. No definitive diagnosis was reached; however, it is not clear that PNPO deficiency (which may present with similar clinical features) was excluded in this case.

Assmann et al. (226) describe an interesting series of patients with L-dopa non responsive dystonia (DND) in whom reduced CSF 5-HIAA was observed; suggestive of reduced central serotonin turnover. In this group of patients central dopamine metabolism was unaffected and potential treatment options are proposed. The underlying molecular mechanism remains to be defined.

1.2.7 Carcinoid syndrome

This clinical entity describes the flushing, diarrhoea and heart disease that occurs when tumours of the enterochromaffin cells in the gastrointestinal tract produce active substances that reach the systemic circulation (227). This usually occurs when hepatic metastases are present. The secretion of large amounts of serotonin is responsible for many of the symptoms including increase in gut motility, bronchoconstriction and fibrotic reactions in the heart and elsewhere. As tryptophan is diverted away from nicotinamide synthesis and towards serotonin production, symptoms of pellagra may develop in some instances.
1.2.8 Serotonin abnormalities in autism

Evidence for altered serotonin metabolism in autism comes from the observation first made some 50 years ago, that whole blood serotonin is elevated in up to one third of patients with autism (228). Since this time, hyperserotonenaemia has been documented in many studies of autistic individuals and serotonin has been shown to be elevated independent of intellectual disability and phenotypic variability (229). The possibility that disrupted serotonin metabolism is implicated in autism is both biologically plausible and appealing given the well described functions of this neurotransmitter.

Functional neuroimaging studies have further advanced knowledge in this field. Serotonin synthesis capacity of the brain has been measured in vivo in two studies using Positron Emission Tomography (PET) with an alpha \(^{\text{[11]}\text{C}}\) methyl-L-tryptophan tracer (230;231). In normally developing children aged 2-5 years there is a period of markedly increased serotonin synthesis (200% of adult values) which is then followed by a decline towards adult values after 5 years of age. In contrast, up until 5 years of age, autistic patients show a reduced capacity for serotonin synthesis which then increases to exceed adult values by the age of 15 years (230-232). PET studies have also revealed focal abnormalities of serotonin synthesis with evidence of cortical asymmetry in autistic patients. Although not surprising it is perhaps disappointing that no consistent abnormality of the serotonin metabolite (5-HIAA) in the cerebrospinal fluid of autism patients has been detected (233).

Animal models of autism also support the hypothesis that serotonin is involved in the disease process. As the immature blood brain barrier in utero allows passage of serotonin, exposure to high levels (via any mechanism) during this period could lead to loss of central serotonergic terminals by negative feedback and hence to development of autistic spectrum disorder (ASD). This is illustrated in an animal model which was developed to mimic the hyperserotonenaemia of autism (234). In this model pregnant rats are administered a serotonin agonist on day 12 of gestation which is continued postnatally in the pups for a varying amount of time. The resulting effect (which mimics the 50% rise in serotonin seen in some autistic patients) eventually leads to loss of central serotonergic terminals in the offspring who have abnormal autistic-like behaviour (altered social behaviour, seizures and...
hyper-responsiveness to sound and touch) (234;235). Following on from this, recent work suggests that prenatal exposure to SSRI’s during the first trimester of pregnancy in humans poses a modest increase in risk for the development of autistic spectrum disorder (236).

The mechanism of elevated whole blood serotonin in autism and an understanding of its implications for the metabolism of serotonin within the central nervous system remains an intriguing and as yet unsolved part of the disorder. The finding needs to be considered against the background of other knowledge of the disorder and its pathogenesis.

1.3 AUTISM

More than 60 years ago the first descriptions of an autistic child were published by Leo Kanner in the United States (237) and Hans Asperger (238) in Austria. The term ‘autistic’, used by both physicians was in fact originally proposed by Bleuler to describe characteristics observed in schizophrenia. He wrote:

“The [...] schizophrenics who have no more contact with the outside world live in a world of their own. They have encased themselves with their desires and wishes [...] they have cut themselves off as much as possible from any contact with the external world. This detachment from reality with the relative and absolute predominance of the inner life, we term autism”.

1.3.1 Diagnosis of autism

Despite significant advances in scientific and clinical research, autism remains a behaviourally defined disorder. The diagnosis is based largely on clinical history, observation and developmental assessments, with parental, teacher and multidisciplinary medical teams all having an important input. Although recognised as a very heterogeneous condition, all individuals affected with autism share a triad of common features; atypical social interaction, delayed and disordered language and a markedly restricted repertoire of activities and interests (239). The range of clinical symptoms demonstrated within these broad descriptive areas is vast, for
example social behaviours can range from an apparent total lack of awareness of others to lack of appropriate eye contact; language problems may vary from a complete lack of verbal speech to the production of speech that is associated with atypical syntax, prosody or intonation. At the ‘high-functioning’ end of the autistic spectrum individuals may be diagnosed with Asperger’s syndrome which specifically requires that the cognitive ability of the child lies within the normal range and that language development is not delayed. Some individuals with autism excel in certain areas having for example exceptional islands of rote memory or isolated talents (239).

The diagnostic criteria in common use come from two main classification systems; the ‘Diagnostic and Statistical Manual of Mental Disorders, DSM-IV’ and the ‘International Classification of Diseases, ICD-10’. In common both state that symptoms in at least some areas should have onset prior to three years of age. Validated instruments used in clinical practice and scientific research to assess or diagnose autism include the Autism Diagnostic Observation Schedule – Generic (ADOS-G), the Autism Diagnostic Interview – Revised (ADI-R) and the Developmental, Dimensional and Diagnostic Interview (240).

1.3.2 Epidemiology of autism

For a long time autism was considered to be a rare disorder but over the last decade this has changed considerably and autism is now reported to have a prevalence of approximately 1% in school aged children (241). This prevalence rate is approximately 20 – 100 times higher than studies performed 40 years earlier and the reason for this demands explanation. Some attribute the apparent increased prevalence to improved ascertainment; a broadening of the diagnostic criteria and improved professional and public awareness. Other schools of thought however consider the observation to reflect the contribution of an environmental factor or factors, and many have been proposed (242).

It is interesting to note that recent studies have described an increased incidence of autistic spectrum disorders in babies born extremely premature compared to the general population (243;244). The distribution of ASD symptoms in this cohort suggests that an increased liability to ASD symptoms impacts many extreme
preterm children rather than a distinct subgroup and that those reaching the threshold for a diagnosis of ASD represent the extreme end of a distribution. Of the factors identified as being independently associated with developing ASD symptoms, potential links with nutrition were highlighted, as not receiving breast milk was found to be associated with the development of ASD symptoms. This is likely to be a complex association and needs further research.

One striking feature of autism which has remained stable over time is the male predominance of the disorder; a male to female sex ratio of 3-4:1 is reported for autism and 8-9:1 for Asperger’s syndrome. This intriguing and robust finding has led some to suggest that autism is an ‘extreme form of male brain’ (245;246) and many hypotheses have sought to understand autism through its gender bias. To date none has been able to fully explain the observation in this very complex disorder.

1.3.3 Associated medical problems

Autism is often accompanied by additional medical problems which may go unrecognised due to the difficulty in clinical assessment and examination of this group of children. These co-morbidities represent a neglected area of research which may provide an interesting and alternative avenue towards understanding the underlying pathology of autism.

Epilepsy occurs in a significant minority of individuals with autism with the prevalence of seizure disorders being estimated at up to 35% in adults (239). A recent study found that epilepsy developed in 22% of autistic individuals followed up to 21 years of age, the majority of which began after 10 years of age (247) and were associated with intellectual disability and female gender. Seizures may be of any type with complex partial being most commonly described and they can usually be controlled with conventional anticonvulsant medication.

Sleep disorders are common and problematic amongst the autistic population with prevalence rates estimated to be between 44 and 83% (248). Poor ‘sleep hygiene’ is likely to exacerbate behavioural problems during the day thus recognition and treatment is important. Difficulty initiating and maintaining sleep are the most commonly reported sleep problems described by parents; other difficulties include
snoring, apnoeas, and nocturnal arousals associated with screaming and sleep walking (239).

Children with autism frequently have problems related to gastrointestinal (GI) dysfunction with a variety of problems being reported including constipation, diarrhoea, food intolerance, abdominal pain and gastro-oesophageal reflux. All of these disorders are common in the normally developing child, therefore, it is difficult to be certain that they are seen with increased frequency in autism. A recent study however found that parents reported significantly more GI problems in their autistic offspring compared to their normally developing siblings (42% compared to 12%). In this study autism severity was associated with a greater likelihood of developing GI problems (249).

It has recently been recognised that many individuals with autism have subtle gross and fine motor dysfunction compared to normally developing children (239). Hypotonia was found to be the most common motor symptom in a cohort of over 150 children examined by Ming et al. (250) and ‘toe walking’ and gross motor delay were also reported. All motor problems investigated appeared to be more prevalent in the younger age groups suggesting an improvement with age; however the aetiology of such observations remains uncertain.

1.3.4 Treatment

Autism is a lifelong neurodevelopmental disorder. Unfortunately the core symptoms of this disabling condition have largely proved to be refractory to pharmacological intervention and therapeutic strategies mainly centre upon educational and behavioural measures which are still at an early stage of development (251). Despite this, many children with autism will receive some form of medication during their development and as the neurochemical basis of autism is largely unknown, treatment with pharmacological agents is essentially empirical (252). Antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs), are the most widely prescribed drug for children with autism (253). Similar to their beneficial role in obsessive compulsive disorder, SSRIs are prescribed to alleviate the repetitive and perseverative behaviours seen in many patients with autism. From a neurochemistry perspective, SSRIs are considered to work by normalising central serotonin
metabolism (254). Convincing benefit of SSRI medication is not forthcoming in the medical literature. While some studies have shown a therapeutic response in terms of repetitive and maladaptive behaviours compared to placebo (255;256) a recent large, multi-centre trial failed to show any improvement with respect to compulsive and repetitive behaviours compared to placebo (257).

Other classes of drug commonly used in the treatment of autism include stimulants (e.g. methylphenidate) and antipsychotics (e.g. risperidone). Both classes have shown improvement in difficult behaviours to some extent in the autistic population but they are often poorly tolerated and none appear to impact upon the central core symptoms of impaired social communication (252).

Encouraging work in animal models has recently demonstrated that some paediatric disorders which are associated with autism such as Rett syndrome, tuberose sclerosis and fragile X can be largely reversed in adulthood by reversal of the underlying genetic defect and thus production of a normal protein (258-260). This is perhaps not wholly unexpected considering that the autistic behaviours observed in children exposed to extreme social deprivation (for example in Romanian orphanages) are reversible upon transfer to a nurturing environment (261). These observations and those in animal studies not only suggest that many of the deranged processes in ASD are not permanent and thus may have a biochemical (hence reversible) rather than structural basis but also, should the findings be generalisable to humans, then an exciting area of future research is opened up.

1.3.5 Genetic risk for autism

There exists compelling evidence that autism has a genetic basis which first became apparent on publication of a study that showed a higher concordance in monozygotic twins than in dizygotic twins (262;263). Prior to this autism had been considered to be a consequence of, amongst other things, bad parenting and poor maternal bonding.

Although decades of research support a significant genetic contribution to the aetiology of autism, high heritability does not imply a simple model of genetic transmission or an easily identifiable major causative gene(s) (264). In fact the findings of recent research have done as much to challenge current paradigms of
neuropsychiatric disease, as to answer questions concerning their aetiology. Some authors now propose that autistic spectrum disorder is best considered as ‘the autisms’ reflecting the fact that ASD is not one condition but many aetiologically distinct forms (264) as current research strongly suggests a significant genetic heterogeneity where the effects of many genes interact with one of many various environmental factors (239).

One of the major problems encountered in unravelling the genetic basis of autism is the fact that diagnosis of the disorder remains a clinical one reflecting the fact that little is known about pathology, hence there is no ‘biomarker’. Similar difficulties are frequently encountered in other neuropsychiatric disorders, such as schizophrenia. Contributing to this, the phenotype of autism is vast and patients’ social disability lies on a continuous spectrum. Attempts to overcome this difficulty by specifically investigating endophenotypes within the broad spectrum may in part help to improve future research.

Adding to this complication, autism is a neurodevelopmental disorder and the disease is very likely to have its onset early in fetal development and early childhood. Research over the past decades suggests that ‘higher-order’ functions that are disrupted in psychiatric disorders emerge from complex neurodevelopmental processes that are guided by thousands of genes (265). A complete understanding of this remains one of the major unmet challenges of scientific research.

The study of Mendelian single-gene disorders has in the past offered useful insights into the molecular mechanisms of autistic behaviours, for example Rett syndrome and Tuberose Sclerosis (266). Some, however, have concerns regarding this approach, arguing there are subtle differences in the social phenotype of syndromic autism compared to idiopathic autism and also that the intellectual disability associated with many genetic syndromes simply increases the risk that autistic behaviours will be revealed (267).

In the pre-genome wide association study (GWAS) era, genetic linkage studies had limited success, with many at first promising results, failing to be replicated in subsequent studies. Genetic regions 7q, 15q, 22q and 2q were the areas most frequently reported as showing an association with autism in more than one study.
Two findings in particular from linkage studies continue to generate interest, including regulatory SNPs in the receptor tyrosine kinase MET gene (270) (271) and SNPs corresponding to the Contactin-associated protein 2 (CNTNAP2) gene (272;273).

The genome wide association approach, facilitated by the development of advanced microarray technology, has been in vogue over recent years and consequently applied to ASD. Progress in this area was initially hindered by the enormous under-powering of early studies. Allelic effect sizes were overestimated and overall, when viewed cumulatively, the common risk alleles reproducibly identified by GWAS have only accounted for a tiny fraction of the anticipated risk for ASD. Similar conclusions have been reached for other common conditions (e.g. diabetes).

Despite these initial difficulties, three disorder-related alleles that meet accepted criteria and which survive internal replication have been identified [a region on 5p between Cadherin 9 and Cadherin 10 (274), Semaphorin 5A (275) and MACROD2 gene (276). Significant uncertainty remains however as to the relevance of these loci because each of these studies has failed to replicate findings of the others. Perhaps more importantly, translating the results into meaningful biological mechanisms which are relevant to the disease is at present lacking (265).

A recent and exciting advance in our knowledge of the genetic causes of autism has come from whole genome DNA microarrays which suggest an important role for structural chromosomal abnormalities. A seminal study by Sebat et al. (277) identified de novo copy number variations (CNV) in 3% of autistic children from multiplex families (with two or more affected members) and in 10% of autistic children from simplex families. The CNVs were composed of deletions in 70% of cases and duplications in 30%; DNA fragment sizes ranged from 160 kb to several megabases, thus containing segments from the size of a single gene to chromosomal regions harbouring many genes. Such variations were found in only 1% of control subjects. Subsequent studies of increasingly large numbers have confirmed initial findings and although the resolution of detection of new technologies has increased the number of de novo CNV has remained fairly constant at 5-10% (265). Some studies have also identified recurrent structural variations that are strongly
associated with ASD (for example, deletions and duplications at 16p11.2, duplications at 15q11-13) all of which reach genome wide significance.

Further work is required to understand how both deletion and duplication of a chromosomal region may result in an identical phenotype and how identical variations may be associated with divergent phenotypes (deletions at 16p11.2 are strongly associated with ASD, intellectual disability and obesity (278); duplications are associated with schizophrenia and ASD (279).

Ultimately ASD and other neuropsychiatric disorders represent conditions whereby genetic mutations in potentially hundreds of different genes converge onto a small number of molecular and anatomical pathways which play a critical role in the development and function of the central nervous system (265). The recent discovery that the FOXP2 protein is a ‘transcriptional regulatory hub’ which has downstream influence on three genes implicated in autism [CNTNAP2 (280), MET (281) and PLAUR (282)] supports this.

Our understanding of autism has come a long way since its first description in 1943 and research into the genetic basis in particular has made remarkable progress over the last 10 years. Many fundamental questions remain unanswered however. In the future, it seems likely that an integrative approach combining top-down and reductionist (bottom-up) genetic processes complemented by multi-disciplinary and computational approaches will allow further advances in the field. Alongside this, a shift in current thinking is required in order to reconceptualise the relationship of genotype and phenotype in ASD (265).

1.3.6 Neuropathology and neuroanatomy

A universal neuropathology or neuroimaging phenotype has not yet emerged in autism. This may reflect the small sample sizes of studies to date or given the heterogeneous nature of ASD, it may be argued that consistency would not be expected (283).

Some features that are repeatedly observed in autistic patients include evidence of early brain overgrowth (manifest as macrocephaly) followed by normalisation or ‘growth arrest’ in later childhood. Brain overgrowth does not appear to be evident in
all brain regions but is most prominent in the frontal lobes and anterior temporal regions (284) and affects both grey and white matter. Various hypotheses have been proposed suggesting that abnormalities in brain growth form part of a developmental disconnection syndrome encompassing brain regions involved in language, social cognition and emotional reciprocity (285). Against this, some groups have found that macrocephaly is actually a familial trait that is seen at a similar rate in macrocephalic ASD probands and their unaffected siblings and parents.

Pathology studies of the autistic brain have revealed cerebellar and brainstem abnormalities with several studies showing a decreased number of cerebellar Purkinje fibres in particular. Subsequent detailed work has also highlighted disruptions in frontal cortical mini-columnar organisation (286). Mini-columns are vital for cortical information processing and abnormalities in their architecture could feasibly result in complex information processing defects seen in autistic individuals. How these observations relate to previously described macrocephaly remains to be resolved.

1.4 SUMMARY AND AIMS

In summary, vitamin B₆ is a critical component of human metabolism whose role as an enzyme cofactor (in the form of PLP) is particularly important for normal neurological function. This is exemplified by several heritable seizure disorders of childhood where various disease mechanisms result in reduced availability of PLP. Such examples include PDE, PNPO deficiency and Hyperprolinaemia type II.

PLP is the enzyme cofactor for aromatic aminoacid decarboxylase (AADC), which catalyses the final reaction in the synthesis of the neurotransmitters serotonin and dopamine. It is feasible that any abnormality on the pathway for production of PLP or in the activity of AADC could result in neurotransmitter disturbance. Indeed this is the case in a primary genetic deficiency of AADC where a severe reduction of both dopamine and serotonin metabolites are observed.

A clear understanding of the pathological basis of autism has long evaded researchers yet biochemical evidence points to an abnormality at some level in the
metabolism of serotonin as up to one third of autistic patients have hyperserotonemia. The metabolic pathways of vitamin B₆ and serotonin are closely linked via the AADC enzyme and further work in this area may advance understanding of the autistic spectrum disorders. Equally further biochemical and genetic investigation of children with undiagnosed seizure disorders responsive to vitamin B₆ may identify new monogenic disorders. Both avenues of research will benefit from the development of new laboratory techniques to assess vitamin B₆ status in patients and this forms the basis of my thesis.
Chapter 2
Materials and Methods
2.1 Materials

The following were purchased from VWR International Ltd (Lutterworth, UK): Disodium hydrogen orthophosphate; sodium dihydrogen orthophosphate; ethylenediaminetetraacetic acid (EDTA); hydrochloric acid (12.2M); methanol HiPerSolv for HPLC; and sodium hydroxide.

The following were purchased from Sigma Aldrich (Poole, UK): 1,4-Dithioerythritol; pyridoxal 5’-phosphate, L-3,4-dihydroxyphenylalanine; perchloric acid; sodium-L-ascorbate; dopamine hydrochloride; 1-octanesulphonic acid; serotonin; trichloroacetic acid; pyridoxal; pyridoxamine dihydrochloride, pyridoxal hydrochloride, pyridoxine hydrochloride, pyridoxamine 5’-phosphate, pyridoxal 5’-phosphate mono-hydrate and 4-pyridoxic acid; acetic acid, heptafluorobutyric acid (HFBA) and trichloroacetic acid (TCA).

The following were purchased from Fisher Scientific UK Ltd (Loughborough, UK): HPLC grade methanol and 850ml/L (85%) orthophosphoric acid HPLC (electrochemical grade).

HPLC vials and caps were purchased from Chromacol (Welwyn Garden City, UK).

Four deuterated vitamers were used as internal standards: Pyridoxal methyl D3 hydrochloride [>98% atom %D] was purchased from Isotec, pyridoxine D2 hydrochloride (5-hydroxymethyl-D2) [>98% atom %D] from CDN Isotopes, 4-pyridoxic acid D2 (5,5’-D2) [>98%] was purchased from Buchem BV and D2 Pyridoxal 5’-Phosphate was kindly supplied as a gift by Professor Coburn, Department of Chemistry, Indiana University, Purdue University, Forte Wayne.
2.2 BIOCHEMISTRY METHODS

2.2.1 Measurement of PLP in plasma and CSF by reverse phase HPLC

PLP was measured by Viruna Neergheen and Marcus Oppenhein in the Neurometabolic Unit, National Hospital using a commercial kit (Chromsystems, Munich, Germany).

200 μL of plasma or CSF was mixed with 300μL of precipitation reagent (Chromsystems) and incubated at 4°C for 10 minutes. Samples were then centrifuged at 12000 x g for 5 minutes at room temperature. 200 μL of supernatant was diluted with 200 μL of neutralisation agent (Chromsystems) and 80 μL of derivatisation reagent (Chromsystems) was subsequently added. Samples were then incubated at 60°C for 20 minutes and subsequently incubated for 4°C for 10 minutes. Samples were then centrifuged for 2 minutes at 12000 x g for 5 minutes at room temperature. 200 μL of supernatant was transferred to the autosampler vials prior to injection onto the HPLC.

The HPLC equipment used was as follows: PU-980 pump (Jasco); AS-950 autosampler (Jasco); and FP-920 fluorescence detector (Jasco). The fluorescence detector was coupled to a computer and data was recorded using AZUR version 4.6 data capture and analysis software (Datalys, Saint Martin D’Heres, France).

The mobile phase (Chromsystems) was run isocratically at a flow rate of 1.3 ml/minute. 50 μL of each sample was injected onto the column (Chromsystems) which was maintained at 20°C. PLP was detected by fluorescence detection with an excitation wavelength of 415 nm. PLP concentration of the sample was quantified against an external plasma calibration standard (Chromsystems) which ranged between; 40 and 60 nM. AZUR software version 4.6 was used for data analysis (95;287).
2.2.2 Measurement of B₆ vitamers and 4-pyridoxic acid by HPLC linked tandem mass spectrometry (LC-MS/MS)

2.2.2.1 Sample collection and preparation

Venous blood samples were taken into EDTA containing tubes and were centrifuged at 7000 x g for 10 minutes at 4 °C within 60 minutes of collection and the plasma was removed immediately. Plasma samples were then stored at -80 °C until analysis.

On the day of sample analysis proteins were precipitated by mixing 60 μL of plasma with an equal volume of 0.3 N TCA (containing deuterated internal standards) to a final TCA concentration of 0.15 N. The sample was vortexed thoroughly for 30 seconds, left on ice in the dark for 60 minutes and finally centrifuged at 7,000 x g for 10 minutes at 4 °C. The resulting supernatant was transferred to a HPLC vial and placed in an autosampler where the samples were kept at 4 °C and protected from light.

2.2.2.2 Determination of B₆ vitamers and 4-pyridoxic acid

LC-MS/MS was performed using a Waters Alliance 2795 LC system linked to a triple quadrupole Micro Quattro instrument (MicroMass, Waters, UK). A HS F5 column (Supelco; 10cm x 2.1 mm; 3 μm) fitted with a HS F5 guard column was used with a mobile phase consisting of 100% methanol, 3.7% acetic acid and 3.7% acetic acid containing 100 mM HFBA at a flow rate of 0.2 ml/minute. The mobile phase gradient table was as shown in Table 4. A 25μL volume of deproteinised plasma was injected every 25 minutes.
Table 4. Gradient profile of the mobile phase for separation of $B_6$ vitamers in plasma using a HSF5 column with HSF5 guard column

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>100% methanol (%)</th>
<th>3.7% acetic acid (%)</th>
<th>3.7% acetic acid &amp; 100 mM HFBA (%)</th>
<th>Flow rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>2.5</td>
<td>95.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>2.00</td>
<td>2.5</td>
<td>95.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>10.00</td>
<td>49.5</td>
<td>48.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>15.00</td>
<td>97.5</td>
<td>0.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>17.00</td>
<td>49.5</td>
<td>48.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>20.00</td>
<td>2.5</td>
<td>95.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>25.00</td>
<td>2.5</td>
<td>95.0</td>
<td>2.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$B_6$ vitamers and PA were detected using multiple reaction monitoring mode (MRM) with the mass spectrometer operated in positive ion mode for all compounds (Table 5). All $B_6$ vitamers and PA could be differentiated on the basis of m/z ratio and retention time. PLP, PMP and PNP had very similar retention times and parent ions that differed by only 1 Da. There was, however, no cross talk between ion pairs originating from these two different analytes.
Table 5. The mass spectral specification for detection of B₆ vitamers in plasma and their deuterated internal standards

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (minutes)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>9.30</td>
<td>168.2</td>
<td>150.0</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>16.10</td>
<td>169.2</td>
<td>133.9</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>11.10</td>
<td>170.2</td>
<td>134.0</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Pyridoxic Acid</td>
<td>7.60</td>
<td>184.2</td>
<td>148.0</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>3.94</td>
<td>248.1</td>
<td>150.0</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Pyridoxamine phosphate</td>
<td>3.89</td>
<td>249.1</td>
<td>134.0</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Pyridoxine phosphate</td>
<td>3.86</td>
<td>250.1</td>
<td>134.0</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>d3 pyridoxal</td>
<td>9.30</td>
<td>171.2</td>
<td>153.0</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>d2 pyridoxine</td>
<td>11.10</td>
<td>172.2</td>
<td>136.0</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>d2 pyridoxic acid</td>
<td>7.60</td>
<td>186.2</td>
<td>150.0</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>d2 pyridoxal phosphate</td>
<td>3.90</td>
<td>250.1</td>
<td>152.0</td>
<td>27</td>
<td>19</td>
</tr>
</tbody>
</table>

[All analytes were detected in positive ion mode]

The linearity, reproducibility and accuracy of this method were fully validated and compared to the HPLC method for measurement of PLP described in Section 4.3. The full details of LC-MS/MS method development and validation are contained in Chapter 4. MassLynx software was used for data acquisition and analysis.
2.2.2.3 Quantification of B<sub>6</sub> vitamers and 4-pyridoxic acid

Stock solutions of B<sub>6</sub> vitamers, 4-pyridoxic acid (PA) and the deuterated internal standards were made with purified deionised water and stored at -80°C. They were placed on ice and protected from the light during laboratory handling.

B<sub>6</sub> vitamers and PA were quantified in plasma by the addition of a known concentration (100 nM) of deuterated internal standard. The same concentration of internal standard was used to construct calibration curves with reference vitamers (except PNP which was not available) and PA. The amount of PLP and PMP present was calculated from the ratio of the signal area for the vitamer to the signal area for d2 PLP. Similarly d3 PL was used to calculate the amount of PL and PM present, d2 PN for PN and d2 PA for PA. PNP was quantitated by determining the ratio of the signal from PNP to the signal from deuterated PLP. This was converted to a concentration in plasma by using the calibration curve for PLP. If the calibration for PNP was identical to that for PLP this would be the plasma concentration of PNP in nmol/L, however we cannot make that assumption and so PNP concentrations are expressed in the results as “concentration units”.

2.2.3 Aromatic L-amino acid decarboxylase (AADC) activity assay in plasma

2.2.3.1 Principle

AADC activity was measured in plasma by Dr George Allen and Dr Emma Footitt. The assay measures the conversion of L-dopa to dopamine by the plasma sample and is thus measuring plasma dopa decarboxylase activity. It is based upon the method described by Hyland and Clayton (288). The sample is initially incubated with the AADC cofactor PLP which is present in excess and the reaction is then initiated by addition of the substrate. It is terminated after a defined incubation time by protein precipitation. HPLC linked to electrochemical detection (HPLC-ECD) is used to quantify the amount of dopamine present which is assumed proportional to the amount of dopa decarboxylase activity.
2.2.3.2 Method for L-dopa decarboxylation

50 μL of lithium heparin plasma was incubated with 25 μL 0.7 mM PLP in assay buffer (500 mM sodium phosphate pH 7.0, 0.167 mM EDTA and 39 mM dithioerythritol) for 120 minutes at 37°C in a shaking water bath.

25 μL 20 mM L-dopa in 6 mM HCl was added to the reaction mixture. After gentle mixing it was incubated for a further 90 minutes at 37°C in a shaking water bath.

The reaction was terminated with 250 μL 0.8 M perchloric acid. Samples were incubated at room temperature for 10 minutes before centrifugation at 12000 x g for 5 minutes at 4°C.

For each plasma sample a ‘substrate blank’ was also prepared and analysed which was treated in an identical manner to the patient sample except that L-dopa was not added during the incubation. Assay buffer was used instead to ensure the final volume was consistent. For each batch a ‘sample blank’ was also prepared and analysed in the same manner except that the plasma sample was omitted from the reaction and assay buffer was instead used to keep the final volume consistent.

2.2.3.3 HPLC-ECD for detection of dopamine

The mobile phase consisted of 50 mM sodium phosphate (pH 3.6), 5 mM octanesulphonic acid, 67 μM EDTA, 43 mM orthophosphoric acid, 230 ml/L methanol in HPLC grade water.

HPLC equipment: PU-2080 Plus pump (Jasco (UK) Ltd., Great Dunmow, UK); AS-2057 Plus autosampler (Jasco); Coulochem III electrochemical detector and 5010 analytical cell (ESA Analytical Ltd., Aylesbury, UK). The electrochemical detector was connected to a computer and data recorded using AZUR Version 4.6 chromatography data capture and analysis software (Datalys).

200 μL of each sample was transferred to a vial, loaded onto the autosampler and maintained at 4°C. The mobile phase flow rate was 1.2 ml/minute and the column temperature was maintained at 25°C.

50 μL of each sample was injected and then separated on a 250 x 4.6 mm x 5 μm HiQSil C18-W column (Kromatekh. Corp. Tokyo, Japan). Dopamine was detected
by coulometric detection. The screening electrode (E1) was set to 20 mV to oxidise analytes with a low oxidation potential. The optimum voltage of the detector electrode (E2) was determined by voltamogram by measuring the peak area of 1000 nM dopamine at various E2 potentials from 50 mV to 400 mV. A potential of 350 mV was selected for detection of dopamine.

Samples were quantified using an external standard of 1000 nM dopamine in 60 mM HCl. Calibration curves have been previously constructed which demonstrate linearity between current amplitude and dopamine concentration across all concentrations from 5 nM to 40000 nM of dopamine (Allen G. Postdoctoral thesis UCL; 2011). A quality control (QC) sample was run with each batch of samples to ensure method reproducibility.

2.2.3.4 Data analysis and calculation of enzyme activity

Dopamine was identified and quantified by the AZUR Version 4.6 software package (Datalys) using the following equation:

\[
\text{Concentration (nM)} = \frac{(\text{sample peak area} / \text{external standard peak area}) \times \text{calibration standard concentration (nM)}}
\]

The concentration of dopamine in the substrate and sample blank was subtracted from the sample concentration to give the final concentration produced during incubation using this equation:

\[
\text{AADC activity} = \frac{(\text{final sample concentration (nM)} \times \text{dilution factor})/ \text{incubation time (min)}}
\]

\[= \frac{(\text{dopamine concentration (nM)} \times 10)}{90}\]

The results were expressed as pmol/min/ml plasma.
2.2.4 Whole blood serotonin

2.2.4.1 Principle

Measurement of whole blood serotonin was undertaken by Dr Iain Hargreaves in the Neurometabolic Laboratory, National Hospital for Neurology and Neurosurgery. The vast majority of serotonin in whole blood is contained in platelets. Following the removal of protein whole blood serotonin was separated using reverse phase HPLC with fluorescence detection and quantified by comparison with an internal standard. The method is based upon that of Joseph and Lofthous (289).

2.4.4.2 Specimen collection, handling and storage

As serotonin degrades quickly, correct methods of collection, handling and storage are critical to achieve valid results.

For each subject, 2 ml of blood was collected by myself into an EDTA tube which contained 5 mg of ascorbic acid (Sigma Alrich, UK) to limit degradation. Following collection the tube was gently inverted, snap frozen at the bedside and transported to the laboratory in liquid nitrogen. Here it was stored at -70°C until analysis.

2.4.4.3 HPLC conditions and instrumentation

The mobile phase was prepared using sodium dihydrogen orthophosphate (0.12M) in ultrapure water and methanol. A flow rate of 0.7 ml/minute was employed for sample analysis.

The HPLC equipment used was as follows: Jasco AS-950 intelligent autosampler; Jasco PU-950 intelligent pump; HPLC Technology ODS 5μ, 250 x 4.6 mm column; Jasco Model FP-920, intelligent fluorescence detector and AZUR data capture system or TSP Chromjet SP4400 series integrator.

Serotonin was detected by fluorescence detection at an excitation wavelength of 294 nm and emission wavelength 335 nm.

2.4.4.4 Preparation of working standard

The working standard was made to a final concentration of 500 nM by dissolving 10.6 mg of serotonin (Sigma Aldrich, UK) in ultrapure water containing 10 mg of
ascorbate. It was kept on ice during laboratory handling and is known to be stable under these conditions for 24 hours. For analysis, 200 μL of standard was placed into a vial and 50 μL injected on to the HPLC column.

2.4.4.5 Preparation and analysis of subject samples and quality control (QC)

As no external quality assurance (QA) scheme exists for this method, an aliquot of blood from the previous run was used as a QC sample and was run with each batch of subject samples to assess reproducibility of the method.

To each subject sample and QC, 2.5 ml of ice cold ultrapure water was added to 1 ml of whole blood and was allowed to stand on ice for 10 minutes. Proteins were precipitated by the addition of 1 ml of 10% (w/v) zinc sulphate (Sigma Aldrich, UK) and leaving the sample on ice for a further 10 minutes. Following this 0.5 ml 1M sodium hydroxide (Sigma Aldrich, UK) was added and the sample allowed to stand on ice for a final 10 minutes before centrifugation at 13000 x g for 2 minutes.

200 μL of the sample supernatant was transferred to a vial and 50 μL injected on to the HPLC column.

2.4.4.6 Quantification of whole blood serotonin in subject samples and QC

The concentration of serotonin was calculated by relating the peak height in the subject sample chromatogram to the peak height in the standard chromatogram using the following equation:

Serotonin concentration = (peak height in sample / peak height in standard) x concentration of standard (nmol/L)

2.2.5 Measurement of 5’-hydroxyindolacetic acid (5-HIAA), homovanillic acid (HVA), 5-methyltetrahydrofolate (5-MTHF) and pterins in cerebrospinal fluid (CSF) by HPLC

These metabolites were analysed in the Neurometabolic Laboratory, National Hospital for Neurology and Neurosurgery by Marcus Oppenheim. The techniques are based on previous published methods.
2.2.5.1 Specimen collection

CSF was frozen at the bedside in liquid nitrogen and stored at -70°C until analysis. The first 0.5 ml was collected into a plain tube for measurement of HVA and 5-HIAA. 5-MTHF and PLP were measured in the second 0.5 ml. The final 1 ml was collected into a tube containing dithioerythritol and diethylenetriamine for pterin analysis.

2.2.5.2 Summary of HPLC methodology

HVA and 5-HIAA were separated and quantified using reverse phase HPLC with electrochemical detection. The analytes are oxidised on the second electrode and the current generated is used to determine their concentrations. The first electrode is used to oxidise other readily oxidisable species in the CSF to minimise the background interference from the biological matrix (290;291).

Pterins were analysed by reverse phase HPLC with fluorescence detection following a previously reported procedure (292). Excitation was 350 nm and emission 450 nm (293).

5-Methyltetrahydrofolate was separated and analysed using reverse phase HPLC with fluorescence detection; excitation 295 nm and emission 355 nm (294).

For each metabolite quantification was based upon comparison to a working standard or calibrator of known concentration.
2.3 MOLECULAR BIOLOGY METHODS

2.3.1 Extraction of genomic DNA from whole blood and fibroblasts

2.3.1.1 Principle

Purification of DNA from whole blood first involves lysing the red blood cells (which do not contain DNA) to facilitate their separation from nucleated white blood cells.

DNA is purified from white blood cells contained in the EDTA whole blood sample by lysing the cells with an anionic detergent in the presence of a DNA stabiliser. The DNA stabiliser works by limiting the activity of DNases that are contained in the cell. Contaminating RNA is next removed by treatment with an RNA digesting enzyme. Other potential contaminants such as proteins are removed by salt precipitation. Finally the genomic DNA is recovered by precipitation with alcohol and dissolved in a buffered solution containing a DNA stabiliser.

2.3.1.2 Method for whole blood and fibroblasts

300 μL of whole blood that had been collected into EDTA-containing tubes was mixed with 900 μL RBC Lysis Solution (Puregene Genomic DNA Purification Kit, Minnesota, USA) and incubated for 1 minute at room temperature. During the incubation the tube was gently inverted. The sample was then centrifuged for 20 seconds at 13000 x g and the majority of clear supernatant removed to leave a white cell pellet in 20 μL of fluid. The sample was then vortexed for 10 seconds to resuspend the cells and 300 μL of Cell Lysis Solution (Puregene) added.

For cultured fibroblasts the initial stage of red blood cell lysis was omitted and 300 μL of Cell Lysis solution was first added to the cultured cells which were suspended in 20 μL of residual fluid. The following procedure was then applied to both whole blood and fibroblast samples.

1.5 μL of RNase A solution (Puregene) was next added to the lysed cells and following gentle inversion the mixture was then incubated in a water bath at 37°C for 15 minutes. The sample was then placed on ice to cool quickly and 100 μL
Protein Precipitation Solution (Puregene) added. Following this the sample was vortexed for 20 seconds and centrifuged at 13000 x g for 1 minute.

The supernatant containing the DNA was then poured into a clean Eppendorf tube which contained 300 μL 100% molecular biology grade isopropanol. The sample was inverted gently 50 times before centrifugation at 13000xg for 1 minute.

The supernatant was poured off leaving the DNA as a white pellet in the tube. It was then washed with 300 μL 70% molecular biology grade ethanol and further centrifuged at 13000 x g for 1 minute.

Finally the supernatant was poured off and the DNA resulting from whole blood or fibroblast extraction was resuspended in 100 μL and 50 uL DNA Hydration Solution (Puregene), respectively. This was incubated at room temperature for 24 hours to ensure the DNA sample was completely re-suspended before being stored at -20°C. This gave a predicted DNA concentration of 100 μg/ml (whole blood) and 200 μg/ml (fibroblasts).

2.3.2 Amplification of genomic DNA of targeted genes by the Polymerase Chain Reaction (PCR)

2.3.2.1 PCR conditions

All of the reagents and reactions were prepared and carried out under sterile conditions in the laboratory.

A typical PCR reaction was carried out in a total volume of 50 μL in 0.5 ml microcentrifuge tubes according to the method described by Saiki et al. (295).

Each reaction contained 100 ng of genomic DNA, 25 pmol of each sense and antisense primer, 5 μL of 10 x NH₄ reaction buffer (Bioline Ltd, London, UK), 5 μL (0.2 mmol/L) dNTP’s [ dATP, dCTP, dGTP and dTTP] (Bioline Ltd.), variable concentration of MgCl₂ (1 – 1.5 mmol/L; see later Chapters for detail) and 0.5 μl (2.5 units) BioTaq DNA Polymerase (Bioline). For each reaction a negative control was prepared containing water instead of template DNA to check for possible contamination. All samples were prepared and kept on ice until transferred to the PCR machine for amplification.
Amplification was carried out on a Veriti 96 Well Therma Cycler (Life Technologies, Applied Biosystems, California, USA).

Typical cycling conditions were 96°C for 5 minutes, followed by 35 cycles of amplification in three stages (1) denaturation of the double-stranded DNA for 30 seconds at 96°C, (2) annealing of primers to the complementary DNA strands at a variable temperature for 30 seconds (see later Chapters for detail of individual genes) (3) extension of the DNA template copy by the 5’ to 3’ activity of Taq DNA polymerase for 30 seconds at 72°C and a final extension at 72°C for 10 minutes to ensure that elongation was complete.

2.3.2.2 Analysis of PCR products by agarose gel electrophoresis

Materials

1 X Tris Borate EDTA (TBE) buffer: 45 mM Tris-HCl, 45 mM boric acid and 10 mM Na₂-EDTA, pH 8.0

Orange loading dye: 2.5 ml dH₂O, 2.5 ml glycerol and 0.1 M EDTA, orange dye (BDH Prolabo chemicals, UK)

The PCR products were analysed by agarose gel electrophoresis to determine the specificity of amplification and size of the product. A 1% (w/v) agarose gel was prepared using 1 gram of agarose (Sigma Aldrich, UK) in 100 ml of 1 x TBE containing 1 mg/ml ethidium bromide (Invitrogen, Life Technologies, USA). The gel was poured into a tray containing a comb to create wells and allowed to set at room temperature for 20 minutes. It was then placed into an electrophoresis tank containing 1x TBE buffer.

5 μL of the PCR product was mixed with 3 μL of the loading dye and loaded into the agarose gel wells. 5 μL of 100 base pair ladder (1 μg/μL) (Invitrogen) was loaded into the first lane to use as a marker of product size.

Electrophoresis was carried out at 80 V for 30 – 45 minutes depending on the size of the PCR product. After electrophoresis, the bands on the gel were visualised on an ultra-violet (UV) trans-illuminator (Chemi Doc, Bio-Rad, Hemel Hempstead, UK) coupled to Quantity One software.
The bands from each PCR product were analysed and if a distinct band for each DNA template was not produced then the reaction conditions were altered to optimise the PCR reaction. The following conditions were changed to optimise the reaction: (1) increasing the annealing temperature of the reaction (2) varying the MgCl₂ concentration.

2.3.3 Sequencing

2.3.3.1 Purification of PCR products

Principle

DNA generated from the PCR reaction was purified to remove any excess enzyme, dNTPs and primers. In particular, Shrimp Alkaline Phosphatase was used to hydrolyse excess dNTPs and Exonuclease was used to degrade residual oligonucleotide primers.

Method

Each 20 μL of PCR product was added to 5 μL of a reaction mix containing 1.5 μL dH₂O, 1 μL Exonuclease I (New England BioLabs), 2μL Shrimp Alkaline Phosphatase and 0.5 μL Shrimp Alkaline Phosphatase dilution buffer (USB Products, Affymetrix, Ohio, USA). The mixture was briefly centrifuged then incubated on the Veriti 96 Well Therma Cycler at 37°C for 15 minutes, followed by 80°C for 15 minutes and held at 4°C until it was stored at -20°C.

2.3.3.2 Sequencing of PCR products using the Sanger method

Principle

DNA sequencing of the purified PCR samples was carried out using a method first described by Sanger in 1977 for which he was later awarded the Nobel Prize. It is also known as the ‘chain termination’ or ‘dideoxy’ method.

In summary, firstly the DNA is denatured and the two complementary strands are separated. The oligonucleotide primer then binds to its complementary template sequence and via the action of DNA polymerase, free nucleotides are incorporated to form a new DNA strand. The enzyme continues to extend until it randomly
incorporates a fluorescently labelled dideoxynucleotide. Each of the four fluorescently labelled dideoxynucleotides is tagged with a different fluorescent dye. As these are chemically altered, the chain elongation is terminated and the DNA polymerase enzyme is removed from the DNA strand. This process is repeated many times over resulting in DNA strands of differing lengths each of which finish with a labelled base.

In order to sort the DNA fragments by size and read the labelled bases, the reaction plate is loaded into a sequencing machine where each sample is transferred into a glass capillary and run in a gel based system. Negatively charged DNA of differing fragment lengths move at different speeds through the gel and can, thus can be sorted by size. Finally the fluorescent tag is excited by a laser and identified as one of four bases which is then represented by a coloured peak. By alignment of the fragments from shortest to longest the DNA sequence can be determined.

The peaks corresponding to the bases in the sequence of the patient samples can then be compared to the normal sequence data for each exon to detect base alterations.

**Method**

3 μL of each cleaned PCR product was mixed with 0.5 μL Big Dye version 1.1 (Applied Biosystems, UK) which was kept on ice during laboratory handling; 1.5 μL sequencing buffer (Applied Biosystems); and 1 μL (5 pmol/μL) of sense or antisense primer, made up to 10 μL with 4 μL of ultrapure water.

The samples then underwent a PCR reaction on the Veriti 96 Well Therma Cycler under the following conditions: 95°C for 2 minutes (1 cycle); followed by 35 cycles of 95°C for 20 seconds; 50°C for 10 seconds and 60°C for 3 minutes. The samples were then stored at -20°C until further processing.

**2.3.3.3 DNA precipitation of sequencing reaction**

**Principle**

Ethanol precipitation effectively removes DNA from an aqueous solution in the presence of positive ions such as Na⁺.
**Method**

2 μL 3M molecular biology grade sodium acetate and 50 μL of 100% molecular biology grade ethanol were added to each PCR product and left to stand at room temperature for 20 minutes after vortexing. Following this the samples were centrifuged at 12000 x g for 40 minutes. The supernatant was discarded and 50 μL of 70% molecular biology grade ethanol added to each sample to wash. The samples were centrifuged at 12000 x g for 10 minutes and the supernatant again removed. Finally the samples were centrifuged at a low speed (1000 x g) upside down on tissue paper to remove any residual sample before each DNA sample was re-suspended in 10 μL of a one in ten dilution of Tris EDTA (TE) Buffer (Promega, Madison, USA).

Samples were then sequenced at York House, Great Ormond Street Hospital, UK on an ABI Sequencer.
Chapter 3
Factors affecting the concentration of pyridoxal 5’-phosphate in cerebrospinal fluid
3.1 INTRODUCTION

Analysis of metabolites in CSF has become an essential part of the investigation of children with neurometabolic disease. In infants with symptoms suggestive of deficient central dopaminergic neurotransmission (severe trunkal hypotonia, choreiform movements of the limbs, occulogyric crises) the analysis of neurotransmitter amine metabolites [homovanillic acid (HVA) and 5-hydroxyindole-acetic acid (5-HIAA)] is important in the diagnostic work up. Measurement of HVA, 5-HIAA, 3-methoxytyrosine (3-MT), neopterin, tetrahydrobiopterin (BH₄) and dihydrobiopterin can indicate a diagnosis of BH₄ synthesis or recycling, tyrosine hydroxylase deficiency or aromatic amino acid decarboxylase (AADC) deficiency. CSF amine metabolite analysis may also be helpful in pointing to a diagnosis of PNPO deficiency or antiquitin deficiency in infants with epileptic encephalopathy. The additional measurement of CSF 5-methyltetrahydrofolate (5-MTHF) can lead to the identification of patients with primary central folate deficiency due to mutations in the gene encoding the folate receptor alpha (296) and to secondary folate deficiency in some patients with mitochondrial disorders (297) and Rett syndrome (298).

It is now a logical extension of the investigation of children with a seizure disorder to include measurement of CSF PLP. It was recently shown that pyridox(am)ine 5-phosphate oxidase (PNPO) deficiency can lead to low levels of PLP in the CSF as well as changes in the CSF suggesting the following effects on neurotransmitter metabolism: reduced synthesis of dopamine (low CSF homovanillic acid [HVA], increased CSF 3-methoxytyrosine), reduced synthesis of serotonin (low CSF 5-hydroxyindole-acetic acid [5HIAA]) and reduced metabolism of glycine and threonine (raised CSF glycine and threonine) (123;124). The principal clinical consequence of PNPO deficiency is a severe epileptic encephalopathy in infancy and a low CSF PLP concentration may prove to be the most consistent marker of this disorder for which specific treatment (administration of PLP) can be extremely effective (123).

PNPO deficiency is not the only cause of a low CSF PLP concentration; it has also been documented in pyridoxine dependent epilepsy (99), including cases due to
mutations in the ALDH7A1 gene encoding antiquitin (113). PLP deficiency can also occur as a result of attack by other endogenous nucleophiles such as Δ¹-pyrroline-5-carboxylate in hyperprolinaemia type II, and exogenous nucleophiles such as isoniazid and penicillamine. Other potential mechanisms leading to the reduction of PLP levels in plasma and CSF include drugs that modify PLP metabolism and dietary B₆ deficiency (1). In experimental animals, knockout of genes involved in the regulation of pyridoxal kinase have been shown to result in low brain PLP and defective synthesis of dopamine (41).

Current understanding of factors affecting CSF PLP is limited and several important questions remain unanswered. Specifically, do any of the following affect PLP concentration in CSF: seizures per se, anticonvulsants, and cessation of normal feeding in an infant with a severe seizure disorder? Furthermore, in view of the fact that L-Dopa containing preparations are widely used in children with neurotransmitter disorders, the influence of this drug on CSF PLP status needs to be considered. Finally, it is known that CSF PLP concentrations are age-dependent (95) so analysis must also bear this in mind. This retrospective review of CSF investigation results addresses these considerations.
3.2 METHODS

3.2.1 Patients and sample collection

This study included 256 CSF samples from 248 patients sent over a 1 year period to the Neurometabolic Unit Laboratory, National Hospital for Neurology & Neurosurgery, Queen Square, London from various UK hospitals.

CSF samples were frozen in liquid nitrogen at the bedside according to standard written instructions and a proforma for clinical information and drug history was completed by the local physician at the time of sampling and returned with the CSF sample. The request form for CSF collection is shown in Figure 10.
CSF Monoamine Metabolites, 5-Methyltetrahydrofolate, Pterins & PLP

To be filled in by requesting clinician/laboratory

NEVER MIX SAMPLES FROM DIFFERENT PATIENTS IN THE SAME LIQUID NITROGEN FLASK

STORE UNUSED SAMPLE TUBES AT 4 °C

Surname:  
Forename:  
Sex: M / F  
DOB:  
Hospital:  
Hospital No:  
Specimen date & Time:  
Consultant:  
ClinicalDetails: ……………………………………………………………………………………………………………………………
Drugtherapy: ……………………………………………………………………………………………………………………………

PLEASE NOTE the above details are essential to allow for the accurate interpretation of results.

Collection Instructions

☐ Number three tubes as 1, 2, 3 and by using a sticky label or permanent marker, include patient details such as surname, first name, date of birth, hospital number, and specimen date.

☐ The first 0.5ml of lumbar CSF should be collected into Tube 1 for HVA and 5HIAA measurements.

☐ The second 0.5ml of lumbar CSF should be collected into Tube 2 for 5MTHF and PLP determination.

☐ The next 1ml of lumbar CSF should be collected into Tube 3 which contains 1mg of each preservative (DTE & DETAPAC), for pterin (neopterin, dihydrobiopterin and tetrahydro-biopterin) analysis.

☐ For Pterin analysis, please confirm preservatives are present (ESSENTIAL)…………………..

ALL CSF samples must be frozen at the bedside on dry ice or liquid nitrogen and transported to the laboratory frozen

Figure 10. Neurometabolic Unit, National Hospital for Neurology and Neurosurgery request form for collection of CSF neurotransmitters
3.2.2 Biochemical Analysis

Neurotransmitter amine metabolites, pterins and 5-MTHF were analysed as described in Section 2.2.5 (Materials and Methods) (291;293). CSF samples underwent only one freeze-thaw episode and blood-stained samples were excluded.

CSF PLP concentration was analysed in the second 0.5ml CSF sample by a commercially available kit for PLP determination (Chromsystems, Munich, Germany) using HPLC with fluorescence detection as described in Section 2.2.5.

3.2.3 Molecular Genetic Analysis - Sequencing of the sulphite oxidase gene (SUOX, ENSG00000139531)

Three patients with a seizure disorder associated with positive urinary sulphite (measured on urinary dipstick) / sulphocysteine (measured by ion exchange analysis) and CSF analysis suggestive of pyridoxal phosphate deficiency were investigated for possible sulphite oxidase deficiency. DNA extracted from whole blood was available for two patients and DNA was extracted from skin fibroblasts for one patient (Puregene Genomic DNA Purification Kit) as described in Section 2.3.1.

Polymerase chain reaction (PCR) primers were designed using the Primer3 design website (http://frodo.wi.mit.edu/) based on SUOX transcript ENST00000394109. The 3 coding exons and exon/intron boundaries of the SUOX gene were amplified using the intronic primers detailed in Table 6. Four pairs of overlapping primers were used to cover Exon 3.

A typical PCR reaction is as described in Section 2.3.2.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Product size (bp)</th>
<th>MgCl₂ conc (mmol/l)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exon 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’-TTCACAAACCCAAGGCATTT-3’</td>
<td>676</td>
<td>1.5</td>
<td>55</td>
</tr>
<tr>
<td>A/S: 5’-CTGTCCCTCCTGGACACACT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exon 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’ AGATCCCCACTTTTCCACCT 3’</td>
<td>581</td>
<td>1.5</td>
<td>55</td>
</tr>
<tr>
<td>A/S: 5’ TGGCTCACTGCAGACTTAGC 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em><em>Exon 3</em> (i)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’ GACAGGGCTTTCTCCATGTTG 3’</td>
<td>596</td>
<td>1.5</td>
<td>61</td>
</tr>
<tr>
<td>A/S: 5’ TTCCGGTGAGAAGATAGG 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em><em>Exon 3</em> (ii)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’ ACCGTGGAGACCTCTGACC 3’</td>
<td>469</td>
<td>1.5</td>
<td>59</td>
</tr>
<tr>
<td>A/S: 5’ GGTCTGAGTCCAGTCCCTC 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em><em>Exon 3</em> (iii)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’ AGTGGGAACAGGAGCCATC 3’</td>
<td>595</td>
<td>1.5</td>
<td>59</td>
</tr>
<tr>
<td>A/S: 5’ TGTCCTCTCCATCCAGCTT 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em><em>Exon 3</em> (iv)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: 5’ CCATCTGTGGACTGGGAGAC 3’</td>
<td>575</td>
<td>1.5</td>
<td>59</td>
</tr>
<tr>
<td>A/S: 5’ GTCTTGGTGAAATGTGCT 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S – sense primer, A/S – antisense primer; conc. – concentration * Exon 3 was covered by four overlapping primer pairs

### 3.2.4 Statistical Analysis

Graphical representation and Kolmogorov-Smirnov tests were used to determine if data were normally distributed. Appropriate parametric (Student’s t-test) and non-parametric (Spearman’s coefficient; Mann-Whitney U; multivariate analysis) tests were then used to compare groups (seizures versus seizure free; anti-epileptic drug
(AED) versus no AED; L-dopa versus no L-dopa) and to investigate correlations (CSF PLP and age; CSF PLP and 5-MTHF; CSF PLP and BH₄). Results were compared to the previously established age reference ranges (299) and a modified age-dependent normal range was established.

3.2.5 Survey of Patients with Low CSF PLP

The modified age-dependent reference range was used to identify patients investigated outside the one year period of retrospective review, who had a low CSF PLP concentration. The clinical and biochemical details of these patients were then reviewed to identify the cause of low PLP.

3.3 RESULTS

CSF PLP and neurotransmitter metabolites (HVA, 5-HIAA, neopterin, BH₄ and 5-MTHF) were analysed in 256 samples from 248 patients; age range 2 days – 51 years (mean 4 years 11 months, median 1 year 10 months) (Table 7).

Patients underwent lumbar puncture for a variety of clinical presentations with seizures and movement disorders being the most common indications. Overall CSF PLP was reduced below the previously established UK reference range in 125 (49%) of samples (Table 7) suggesting that the reference ranges may need revision.
Table 7. Characteristics of the cohort in which CSF PLP was measured and of the subgroup in which CSF PLP is reduced below current UK age related reference ranges

<table>
<thead>
<tr>
<th>NUMBER SAMPLES</th>
<th>CSF PLP measurement</th>
<th>CSF PLP reduced below current UK age related reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>256</td>
<td>125</td>
</tr>
<tr>
<td>&lt;3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(44-89 nmol/L)*</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>4 months – 2 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(23-87 nmol/L)*</td>
<td>79</td>
<td>17</td>
</tr>
<tr>
<td>&gt;2 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(25 –40 nmol/L)*</td>
<td>132</td>
<td>83</td>
</tr>
<tr>
<td>Number of patients</td>
<td>248</td>
<td>120</td>
</tr>
<tr>
<td>Gender</td>
<td>152 M</td>
<td>85 M</td>
</tr>
<tr>
<td></td>
<td>96 F</td>
<td>35 F</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>4y 11m (2d -51y)</td>
<td>6y 9m (2d – 51y)</td>
</tr>
<tr>
<td>Clinical history available</td>
<td>246</td>
<td>116</td>
</tr>
<tr>
<td>Drug history available</td>
<td>162</td>
<td>75</td>
</tr>
<tr>
<td>SEIZURES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>116</td>
<td>55</td>
</tr>
<tr>
<td>MOVEMENT DISORDER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>41</td>
</tr>
</tbody>
</table>

*Age dependent reference range shown in italics in parentheses; M – male; F – female; y – years; m – months; d-days
3.3.1 Effect of Age

In keeping with previous reports (95), a negative correlation of CSF PLP with age is observed ($r = -0.51; p < 0.0001$) (Figure 11) and based on this previous UK reference ranges have been established according to age (95).

Figure 11. Correlation of CSF PLP with age for all cases <16 years of age where a drug history was available

Patients on $B_6$ supplementation and where an inborn error of $B_6$ metabolism had been diagnosed were excluded ($r = -0.51, p < 0.0001$)
Our data were compared to current UK and Spanish reference ranges that had been previously published (95). The following were excluded from the analysis; i) cases on supplemental vitamin B₆, ii) cases where no drug history was available, iii) cases with an inborn error in metabolism known to affect CSF PLP, and iv) three patients described below (iv, v and vi), one whose seizures responded to PLP supplementation and two who had abnormal amine metabolites (Table 9).

When analysed in four age groups [< 30 days (A), 1 – 12 months (B), 1 – 3 years (C), >3 years (D)] the data from this study were very similar to the Spanish data across three age groups (Groups B, C & D) (Table 8 and Figure 12). However with the larger numbers in Groups B, C and D in this study, a slightly greater standard deviation was seen. A statistically significant difference was seen between Group B & C (p=0.004) and Group C & D (p=0.04). In the youngest age group (Group A) the group mean, median and lower limit observed in this study were lower than the Spanish data.

Table 8. Statistics by age group of CSF PLP concentration for all cases.

<table>
<thead>
<tr>
<th>CSF PLP (nmol/L)</th>
<th>Group A 0-30 days</th>
<th>Group B 1–12 months</th>
<th>Group C 1–2 years</th>
<th>Group D &gt;3 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data from current study</td>
<td>n = 7</td>
<td>n = 37</td>
<td>n = 28</td>
<td>n = 49</td>
</tr>
<tr>
<td>Range</td>
<td>26 - 69</td>
<td>10 - 136</td>
<td>8 - 86</td>
<td>8 - 41</td>
</tr>
<tr>
<td>Mean (sd)</td>
<td>42.3 (14.3)</td>
<td>42.8 (24.7)</td>
<td>28.8 (15.9)</td>
<td>22.0 (8.0)</td>
</tr>
<tr>
<td>Data taken from Ormazabal et al. 2008</td>
<td>n = 7</td>
<td>n = 16</td>
<td>n = 18</td>
<td>n = 39</td>
</tr>
<tr>
<td>Range</td>
<td>32 – 78</td>
<td>24 – 87</td>
<td>14 – 59</td>
<td>11 – 40</td>
</tr>
<tr>
<td>Mean (sd)</td>
<td>51.5 (16.6)</td>
<td>43.1 (19.3)</td>
<td>30.5 (11.1)</td>
<td>20.7 (6.9)</td>
</tr>
</tbody>
</table>

Comparison is made with Barcelona data from Ormazabal et al. (95)
Table 9. Cases < 30 days of age with low CSF PLP who were excluded from analysis for calculation of reference range

<table>
<thead>
<tr>
<th>Case number</th>
<th>CSF PLP (nmol/L)</th>
<th>Age (days)</th>
<th>Clinical details</th>
<th>Monoamine neurotransmitter and other diagnostic information</th>
<th>Medications; nutrition (where known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>4</td>
<td>14</td>
<td>Antiquitin deficiency</td>
<td>Grossly elevated 3-MT and urinary α-AASA</td>
<td>Phenobarbitone. Expressed breast milk &amp; formula fed.</td>
</tr>
<tr>
<td>ii</td>
<td>5</td>
<td>12</td>
<td>MoCoF deficiency</td>
<td>Reduced HVA &amp; 5HIAA.</td>
<td>Phenobarbitone, phenytoin, PLP, calcium folinate, biotin. Expressed breast milk</td>
</tr>
<tr>
<td>iii</td>
<td>9</td>
<td>2</td>
<td>Antiquitin deficiency</td>
<td>Grossly elevated 3-MT and urinary α-AASA</td>
<td>Phenobarbitone.</td>
</tr>
<tr>
<td>iv</td>
<td>9</td>
<td>21</td>
<td>PLP responsive seizures</td>
<td>Normal monoamines. Urinary α-AASA negative. PNPO no mutations.</td>
<td>Phenobarbitone. Breast fed; feeding difficulties</td>
</tr>
<tr>
<td>v</td>
<td>9</td>
<td>21</td>
<td>Hypertonia, hyper-reflexia. Subsequent good developmental progress</td>
<td>Increased HVA &amp; 5HIAA. Normal ratio.</td>
<td>Domperidone, ranitidine. Regular feeds Formula fed.</td>
</tr>
<tr>
<td>vi</td>
<td>10</td>
<td>20</td>
<td>Hypertonia, abnormal movements</td>
<td>Increased HVA &amp; 5HIAA. Normal ratio.</td>
<td>Clobazam</td>
</tr>
</tbody>
</table>
Following analysis of these larger numbers from our centre, a revision of the age dependent reference ranges was instituted for the four age groups:

<table>
<thead>
<tr>
<th>AGE GROUP</th>
<th>CSF PLP (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Under 30 days</td>
<td>26 - 69</td>
</tr>
<tr>
<td>B. 30 days – 12 months</td>
<td>14 – 92</td>
</tr>
<tr>
<td>C. 1 – 3 years</td>
<td>11- 64</td>
</tr>
<tr>
<td>D. &gt; 3 years</td>
<td>10 – 37</td>
</tr>
</tbody>
</table>

For Groups B, C and D the lower and upper reference limit represent the 2.5\(^{th}\) and 97.5\(^{th}\) percentile respectively. For Group A an observed reference range is used due to smaller sample size (Table 8 & Figure 12).
Figure 12. Distribution of CSF PLP by age group

There is no statistical difference between Groups A & B. A significant difference is seen between Groups B & C (p=0.004) and Groups C & D (p=0.04); ns = not significant.

In Group A six patients were excluded from the reference group analysis, details are listed in Table 9. Patients i) and iii) had antiquitin deficiency. Patient ii) had molybdenum cofactor deficiency with persistently elevated levels of sulphite in the urine; sulphite is one of the nucleophiles known to be able to react with PLP (300) (Figure 13). The remaining cases, (iv) – (vi), were not typical of previously described inborn errors of vitamin B₆ metabolism, however, case (iv) had a seizure disorder responsive to PLP and cases (v) & (vi) have very similar clinical and biochemical phenotypes which could be related to abnormal vitamin B₆ and neurotransmitter amine metabolism.

Of note, the three cases shown to have inborn errors of metabolism [(i), (ii) and (iii)] had the lowest values of CSF PLP in this age group as shown in Table 9.
3.3.2 Effect of seizures, anti-epileptic drugs (AED) and L-Dopa

All cases (excluding those receiving B₆ supplementation, those with confirmed inborn errors of B₆ metabolism and those in whom a drug history was not available) were statistically compared by age group for various factors which may theoretically affect CSF PLP concentration using Student t-test or Mann Whitney U (depending upon data distribution).

No statistical difference was observed for any age group between cases with and without seizures and cases receiving and not receiving anti-epileptic medication (Figure 14). Although statistical significance is not achieved (in part due to small numbers) a trend towards a reduced CSF PLP concentration is seen in patients prescribed L-Dopa in this series. 2 out of 12 patients referred over the 1 year period on L-dopa therapy who had CSF PLP levels which fell below the revised reference range compared to 6 out of 201 not receiving L-dopa.
Figure 14. Comparison of CSF PLP by age group in children with and without seizures (Graph A) and in those receiving and not receiving antiepileptic drugs (AED) (Graph B). No significant difference is observed for any age group.
3.3.3 Relationship between PLP and 5-MTHF in CSF

Figure 15 illustrates a positive correlation between CSF PLP concentration and 5-methyltetrahydrofolate (5-MTHF). This correlation remained significant ($r^2 = 0.167$, $p=0.002$) even after adjusting for the effect of age.

Figure 15. Positive correlation of CSF PLP and 5-MTHF in all cases where a drug history was available, excluding cases taking vitamin B₆ supplementation
3.3.4 Relationship between PLP and BH₄ in CSF

Figure 16 illustrates a positive correlation between CSF PLP concentration and tetrahydrobiopterin (BH₄). This correlation remained significant ($r^2=0.193$, $p=0.023$) even after adjusting for the effect of age.

Figure 16. Correlation of CSF PLP and tetrahydrobiopterin (BH₄) in all cases where a drug history was available, excluding cases taking vitamin B₆ supplementation
3.3.5 Survey of patients with CSF PLP below the revised lower reference limit

The survey identified a further patient with molybdenum cofactor deficiency who had a low CSF PLP concentration. In addition three patients with an undiagnosed seizure disorder presented with positive urinary sulphite or sulphocysteine with normal plasma urate and urine purine analysis, initially suggestive of isolated sulphite oxidase deficiency (Table 10). Of particular note in these patients was the fluctuation over time of sulphite and sulphocysteine excretion. These patients had normal urinary α-amino-adipic semialdehyde (α-AASA) excluding ALDH7A1 deficiency; PNPO gene mutation analysis excluded PNPO deficiency in all patients.
Table 10. Clinical and biochemical details of two patients with genetically confirmed molybdenum cofactor deficiency (P1 & P2) and three patients with an undiagnosed seizure disorder who have a similar biochemical picture (P3, P4 & P5)

<table>
<thead>
<tr>
<th></th>
<th>MoCoF Deficiency</th>
<th>Undiagnosed patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
<td>P2</td>
</tr>
<tr>
<td>AGE</td>
<td>1 mo</td>
<td>3 mo</td>
</tr>
<tr>
<td>ETHNICITY</td>
<td>Arab</td>
<td>Jewish Israeli</td>
</tr>
<tr>
<td>CLINICAL</td>
<td>Neonatal onset severe seizure disorder; infantile onset with dystonia and development arrest</td>
<td>Severe epileptic encephalopathy onset at 10y 5mo; resistant to multiple anticonvulsant medications, requiring Thiopentone infusion</td>
</tr>
<tr>
<td>CSF PLP (age dependent)</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>CSF HVA (324-1098)</td>
<td>120</td>
<td>69</td>
</tr>
<tr>
<td>CSF 5-HIAA (199-608)</td>
<td>20</td>
<td>219</td>
</tr>
<tr>
<td>CSF BH4 (27 – 105)</td>
<td>#</td>
<td>20</td>
</tr>
<tr>
<td>Urine sulphite/sulphocysteine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Plasma urate</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Taurine</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Medications received potentially containing sulphites</td>
<td>#</td>
<td>#</td>
</tr>
</tbody>
</table>

mo – months; y- years; MoCoF – molybdenum cofactor; reference range shown in italics in parentheses +; metabolite present; # not measured/not known; ↑ increased above upper reference limit; ↓ - below reference limit; N - within normal range. Numbers outside the normal range are shown in bold. [Note P1 and P5 are previously reported as (ii) and (iv) respectively in Table 9]
Although atypical in clinical presentation, a primary disorder of sulphite oxidase deficiency was an important differential diagnosis in these patients (P3, P4 & P5). Sequence analysis of the SUOX gene revealed two single nucleotide changes present in all three patients and these were compared to genetic databases. The first is a heterozygous base change C/T within Exon 3 of the SUOX gene (Figure 17), i.e. c.801C>T; p.Asn266Asn. It has not been reported previously and leads to synonymous change [AAC → AAT; Asparagine (N) → Asparagine (N)] and therefore is not expected to affect enzyme activity. This change was not predicted to create an alternative splice site according to a splice site predictor tool (www.fruitfly.org).

![Figure 17. A novel heterozygous single nucleotide change (C/T) in Exon 3 of the SOUX gene in patients P3, P4 & P5 and not in control DNA](image)
The second sequence variation detected was a homozygous change which has been reported previously to be a single nucleotide polymorphism (SNP) [rs773115] in the Ensembl database (www.ensembl.org), c.801C>T; p.Asn266Asn. It is present as cytosine (C) in all patients and results in a synonymous change [TCG → TCC; Serine (S) → Serine (S)] (Figure 18). Information from the ‘1000 genomes’ project suggests that this is the most common variant seen in European (CEU) and Chinese and Japanese (CHB JPT) populations where it is present in >90% of individuals. In the Nigerian population (YRI) it is evident in only 26%. No information is available for Middle Eastern and Jewish Israeli populations.

Figure 18. A homozygous single nucleotide polymorphism rs773115 (G/C/T) in Exon 3 of the SOUX gene in patients P3, P4 & P5 and not in control DNA
3.3.6 Plasma: CSF PLP ratio

Two neonates with CSF PLP reduced below the lower reference limit had normal or elevated plasma PLP resulting in an elevated plasma: CSF ratio (8.2 and 55). These cases share a similar phenotype of a neonatal seizure disorder responsive to treatment with B₆ (pyridoxine or PLP). Neonatal hypophosphatasia, antiquitin and PNPO deficiency were excluded. Over the 1 year period of study, CSF and plasma samples were taken simultaneously in 14 patients to measure PLP in our laboratory. Five cases were excluded as they were taking B₆ medication or because a medication history was not available (Table 11). The plasma: CSF PLP ratio ranges from 0.77 – 4.35; age range 21 days to 18.7 years (Table 11).

Table 11. Plasma: CSF pyridoxal 5'-phosphate ratios measured over the 1 year study period in patients who are not taking B₆ medication

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Plasma pyridoxal phosphate (nmol/l)</th>
<th>CSF pyridoxal phosphate (nmol/l)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Reference range 15 – 73]°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Truncus arteriosus, seizures and dystonia</td>
<td>49</td>
<td>64</td>
<td>0.77</td>
</tr>
<tr>
<td>Refractory epilepsy, Developmental delay</td>
<td>20</td>
<td>20</td>
<td>1.00</td>
</tr>
<tr>
<td>Seizures</td>
<td>12</td>
<td>9</td>
<td>1.30</td>
</tr>
<tr>
<td>Seizure disorder</td>
<td>72</td>
<td>26</td>
<td>2.70</td>
</tr>
<tr>
<td>Epilepsy, developmental delay</td>
<td>46</td>
<td>16</td>
<td>2.80</td>
</tr>
<tr>
<td>Tics, learning disability, progressive myelopathy</td>
<td>54</td>
<td>18</td>
<td>3.00</td>
</tr>
<tr>
<td>Neonatal seizure disorder</td>
<td>94</td>
<td>26</td>
<td>3.60</td>
</tr>
<tr>
<td>Developmental regression, irritable</td>
<td>291</td>
<td>71</td>
<td>4.09</td>
</tr>
<tr>
<td>Developmental delay, progressive dystonia and short stature</td>
<td>87</td>
<td>20</td>
<td>4.35</td>
</tr>
</tbody>
</table>
3.4 DISCUSSION

Measurement of PLP concentration in CSF increasingly forms part of the work up for children with complex neurological disease. However there remains much to be learned about the concentration of CSF PLP in healthy children and adults and how this is influenced by disease processes and medications. Practical factors which may affect CSF PLP concentration should also be considered. For example, vitamin B₆ is a photosensitive compound which will degrade with light exposure therefore samples for PLP measurement should be protected from UV light. In addition PLP is stable when stored at -80°C but may degrade at higher temperatures and on repeat freeze-thawing (301).

In this survey we have reviewed CSF PLP concentration in a large number of cases and examined the effect of age, seizures and various medications. Given the retrospective nature of this review, some shortfalls exist due to incomplete clinical and medication history. This places limitations upon the conclusions which may be drawn, however this is a relatively large series for which methods of analysis were standardised and samples processed within a single laboratory.

The first observation in this series is the large number of cases in whom CSF PLP concentration fell below the previously proposed UK age related reference range. New reference intervals have therefore been calculated based on current data (which represents a larger patient cohort than those reported previously), supported by clinical information. To the best of current knowledge, children in whom reduced CSF PLP suggests a specific diagnosis such as PNPO deficiency or antiquitin deficiency, present with seizures in the early neonatal period (under 30 days). New lower reference limits of CSF PLP concentration for Group A (26 nmol/L) are considered sensitive enough to detect such diagnoses, as evidenced by the cases in this series and those reported previously in the literature (95;113). In addition these particular inborn errors of metabolism are often associated with characteristic CSF neurotransmitter and amino acid profiles which aid diagnosis.

A wide range for CSF PLP concentration is seen in infants aged 1 - 12 months (Group B). It is likely that this accurately reflects infants in this age group who require lumbar puncture for the investigation of neurological problems. The group is
likely to include some infants receiving breast milk only, some receiving formula only and some milk plus solids, therefore $B_6$ intake may vary considerably which could explain the variation. Additionally, some infants could not be fed at all at the time of the lumbar puncture because of intractable seizures for example.

3.4.1 Epilepsy/seizure disorders

Although CSF PLP concentrations in idiopathic epilepsy have not been reported previously, CSF neurotransmitter profiles have been studied with inconsistent conclusions. Duarte et al. (302) prospectively studied CSF pterins and neurotransmitters alongside clinical, EEG and MRI data in 23 infants with severe epileptic encephalopathies. No consistent pattern of abnormality was observed, however elevated neopterin (implying an inflammatory process) in four patients was found to correlate with a poor prognosis. Echenne et al. (303) did not demonstrate any dysfunction in the main neurotransmitter pathways in an older cohort of 37 children on a variety of AED.

When inborn errors causing seizure disorders known to affect PLP concentrations were excluded, statistical analysis of our data suggested that the presence of a seizure disorder does not influence CSF PLP concentration. By the nature of this review however it was not possible to look at idiopathic epilepsies individually, although this may be interesting to pursue.

3.4.2 Medications

3.4.2.1 Antiepileptic drugs (AED)

Most children with a seizure disorder are prescribed AED and it is known that several AED can cause low plasma PLP [(158;304;305) and Section 1.1.14.4]. Hence theoretically AED may also reduce CSF PLP. In this series however, a comparison of children taking AED to those who are not, revealed no significant difference in CSF PLP concentrations (Figure 14). This review is limited in that it was not possible to examine the potential effect of individual AED (too few patients are on single agents) and this may be relevant as mechanisms of PLP reduction are only proposed for certain agents.
Several AED (phenytoin, phenobarbitone, carbamazepine and primidone) are potent hepatic inducers of cytochrome P<sub>450</sub> and other enzyme systems. For these medications it is possible that induction of enzymes involved in the catabolism of PLP leads to reduced plasma PLP concentrations. Many new AED are not hepatic enzyme inducers however and little evidence exists regarding their effect upon PLP in long term treatment.

3.4.2.2 L-DOPA

PLP forms a Schiff base complex (tetrahydroisoquinoline compound) with L-dopa rendering both L-dopa and PLP functionally inactive (Figure 20) [(147;306) and Section 1.1.14.2]. Reduced plasma PLP has been reported in patients with Parkinson’s disease (149) and it may be considered that vitamin B<sub>6</sub> supplementation during L-dopa treatment is necessary in a variety of patient groups (Parkinson’s disease, disorders of pterin synthesis and regeneration and tyrosine hydroxylase deficiency) to prevent secondary AADC deficiency resulting from depletion of its cofactor PLP (307).

Although no statistical difference in CSF PLP is demonstrated, cases on L-Dopa in this series do show a trend to lower CSF PLP concentrations and clinicians should be aware of the potential for B<sub>6</sub> deficiency in patients treated with L-dopa.
Figure 19. Sulphite reactivity with Quinonoid Dihydrobiopterin

Figure 20. Formation of an addition compound between pyridoxal phosphate (PLP) and L-dopa rendering both compounds functionally inactive
3.4.3 Correlation between CSF concentrations of PLP and 5-MTHF

Statistical analysis showed a correlation between the CSF concentration of PLP and that of 5-MTHF across the whole patient series, after adjusting for the effect of age. There are a number of possible causes for this correlation. It could reflect nutritional intake; a diet low in folate is likely to also be lacking in B₆. Many children with neurological disorders have feeding difficulties and therefore their intake of both vitamins may be suboptimal. However, it is also possible that the link is caused by nucleophile and/or free radical stress. It is the reaction between PLP and nucleophiles that leads to the low CSF PLP in antiquitin deficiency, hyperprolinaemia type II and, probably, in molybdenum cofactor deficiency. Folate species can also be attacked by nucleophiles including sulphite (308). PLP and 5-MTHF can also both be attacked by oxygen-derived free radicals (7;309). As the concentrations of alternative free radical scavengers in CSF are relatively low this raises the question of whether nucleophile and/or free radical stress could be implicated in a wide range of neurological disorders of childhood.

3.4.4 Correlation between CSF concentrations of PLP and tetrahydrobiopterin (BH₄)

Similar to the correlation described above, a correlation between CSF PLP and BH₄ concentration was observed across the whole series. These parallel changes may reflect the property of both molecules to act as antioxidants and free radical scavengers as observed in inflammatory disease such as rheumatoid arthritis (310-313).

Some cases in this series have CSF PLP reduced below the revised age related reference range in the absence of a clear contributing diagnosis. In the following section several hypotheses are considered.

3.4.5 CSF PLP in disorders of sulphite accumulation

Alternative mechanisms of PLP reduction in the CSF may involve inactivation of the cofactor by interaction with other molecules, analogous to that described in antiquitin deficiency (1). CSF analysis in two genetically diagnosed patients with molybdenum cofactor (MoCoF) deficiency showed reduced PLP, tetrahydrobiopterin (BH₄) and
monoamine metabolites (Table 10) suggestive of a mechanism of sulphite toxicity which may lead to CSF PLP inactivation and explain some aspects of the neurological phenotype.

Molybdenum is an essential cofactor for 3 enzymes in man; xanthine dehydrogenase (E.C.1.2.1.37), sulphite oxidase (E.C.1.8.2.1) and aldehyde oxidase (E.C.1.2.3.1). Patients with MoCoF deficiency are deficient in the activity of all three enzymes. The clinical phenotype is characterised by a severe seizure disorder with neonatal onset, dystonia and developmental delay often resulting in death in early childhood. Sulphite oxidase deficiency produces an identical clinical phenotype and this enzyme catalyses the final step in the catabolism of sulphur containing amino acids by oxidising sulphite to sulphate (Figure 21).

![Catabolic pathway of cysteine to sulphate illustrating the action of sulphite oxidase](image)

**Figure 21. Catabolic pathway of cysteine to sulphate illustrating the action of sulphite oxidase**

A genetic deficiency of sulphite oxidase results in multiple biochemical abnormalities including elevated sulphite, S-sulphocysteine, thiosulphate and taurine and reduced production of sulphate. MoCoF deficiency shares identical biochemical abnormalities but, in addition, due to dysfunction of xanthine oxidase, plasma urate is reduced and there is an increased excretion of xanthine and hypoxanthine (314).

The underlying disease process is poorly understood, however, toxic accumulation of endogenous sulphite is the most likely pathological mechanism in both disorders. How sulphite may affect neuronal function is unknown; recent work in cell culture suggests that sulphite can inhibit glutamate dehydrogenase, this results in an energy
(ATP) deficiency due to reduced NADH flux through the mitochondrial respiratory chain and thus neurological damage (315).

Sulphite is known to react with numerous biomolecules, essentially acting as a nucleophile attracted to electrophilic centres (316). As sulphite reacts readily with aldehyde groups to produce sulphonates it may be hypothesised that low CSF PLP observed in MoCoF deficiency patients is due to the formation of a sulphite addition compound with the aldehyde group of PLP, rendering it inactive as a cofactor (300) (Figure 13). Sulphite also reacts with quinonoid dihydrobiopterin (qBH₂) leading to reduced levels of BH₄ (317) (Figure 19). Reduced levels of PLP and BH₄ in the brain may lead to a seizure and movement disorder.

Of interest, it has recently been observed that α-AASA is elevated in MoCoF and sulphite oxidase deficient patients and this may, in part, be due to sulphite directly interacting with the aldehyde moiety of α-AASA. However sulphite also inhibits the enzyme required for the metabolism of α-AASA, α-aminoadipic semialdehyde dehydrogenase (112).

Mutational analysis of the SUOX gene in three patients with unexplained accumulation of sulphite revealed two single nucleotide changes present in all three patients, both of which result in synonymous coding. Although one is reported as a single nucleotide polymorphism the other is novel and as it lies within the coding region of SUOX requires further consideration. Recently it has been suggested in the literature that exonic changes, independent of their effect on the encoded amino acid, may be disease causing by disrupting the regulatory elements involved in directing splicing machinery to the correct sites (318;319). Exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) are cis-acting elements present within the coding regions of genes. ESE initiate splicing and serve as a binding site for various splicing factors such as the serine/arginine-rich (SR) proteins. Less is known about ESS, but they are thought to suppress splicing by binding proteins from the heterogenous nuclear ribonucleo-protein family (hnRNP). Such elements also exist within intronic sequences. A careful balance exists between splicing enhancers and splicing silencers which, alongside the strength of the splice site, determine the creation of exon boundaries and formation of mRNA. A change in the nucleotide code may disrupt or create either an ESE or ESS and thus affect pre-mRNA splicing; it has been
predicted that up to 50% of variations within the coding region may have an effect on the protein in this way (320). Although many examples exist [including phenylalanine hydroxylase (318); spinal muscular atrophy 2, SMN2 (319)] it is thought that often such ‘silent’ changes are under-recognised or ignored by researchers who tend to focus only upon variations within the genomic DNA sequence that change the protein sequence (320).

Given the vulnerability of exon splicing to sequence changes, and where no alternative explanation is evident, future work to explore the sequence change found in the patients described above could include use of further splice site or ESS predictor software tools. Other family members and controls of matched ethnic background should also be screened for the change. For thorough assessment, laboratory work to follow up could include synthesis of cDNA from patient mRNA using reverse transcriptase and sequence analysis of the cDNA. This would also enable us to ascertain whether there were any deep intrinsic changes in these patients that would not have been identified using the methodology described.

In the patients reported here, if it is considered that an underlying genetic abnormality does not account for endogenous sulphite accumulation, an alternative mechanism of sulphite accumulation needs to be considered such as an exogenous source. Common exogenous sources of sulphite include its use as a food preservative (e.g. in dried fruits or wine) and as a stabiliser in some drugs. More than 100 medication preparations contain sulphite (321;322). The majority of these are drugs for parenteral administration such as inotropic agents and antibiotics but several are oral preparations. The safe upper level for plasma concentration of sulphite is not known but many adverse effects have been reported after exposure to exogenous sulphites. Although the majority are allergy-related (322;323), development of seizures has been related to administration of morphine containing sodium bisulphite (324) and in vitro evidence for the neurotoxicity of dexamethasone preserved with sulphites has been demonstrated in neural cell culture (325). Cambonie et al. (326) found elevated urinary cysteine excretion in neonates with septic shock compared to well neonates which correlated with severity of the illness (although not to neurological outcome). They showed that cysteine excretion was indirectly related (via non enzymatic sulphitolysis) to the amount of sulphite administered by drug
infusions. Some of these children also had positive urine sulphite test strips and mildly elevated sulphocysteine excretion.

Taurine elevation was present in all patients presented here (Table 10) and this may provide an alternative explanation for elevated sulphite; if there was increased flux through catabolic pathways of sulphur-containing amino acids, elevated sulphite may be seen in association with elevated taurine (which is often increased secondary to tissue breakdown).

Patients P3 - 5 detailed in Table 10 were receiving medication(s) that may have contained sulphite preservatives and this may explain their increased urine sulphite and sulphocysteine excretion and its intermittent nature. In P5 seizure control improved with pyridoxal phosphate treatment which was successfully weaned by 1 year of age. He is now seizure free off medication and developing normally. This child had low plasma PLP status (probably associated with inadequate nutrition) which in combination with sulphite containing medication may have resulted in a B₆ deficiency state. P3 showed some improvement in her movement disorder on L-dopa therapy but succumbed to a severe seizure disorder at 18 months of age. Vitamin B₆ treatment was not trialled. Positive voltage gated potassium channel antibodies (associated with a diagnosis of autoimmune limbic encephalitis) have subsequently been detected in P4 which may explain his seizure disorder but not the abnormal biochemistry (sulphite and monoamine metabolites).

By inactivation of PLP, use of sulphite containing drug preparations may increase the risk of seizures in vulnerable patient groups such as those who have a reduced seizure threshold due to their primary pathology or those who have other risk factors for reduced PLP e.g. poor nutrition. This highlights the importance of measuring plasma and CSF PLP in children with seizure and movement disorders and particularly in those with genetic disorders resulting in sulphite accumulation. Where possible, alternative drug preparations should be used and a treatment trial with vitamin B₆ may be appropriate. Clinicians should also be aware that patients receiving sulphite containing medications may have positive urinary sulphite tests leading to a spurious diagnosis of sulphite oxidase deficiency.
3.4.6 Neonatal seizure disorder with increased plasma: CSF PLP ratio

Shin et al. (99) reported that the plasma PLP: CSF ratio is 2.7 or less in normal individuals of all ages. Data presented here from paediatric patients with neurological disease suggest a higher upper limit of 4.4 (Table 11). Two patients with a seizure disorder of unknown aetiology with a ratio above 4.4 are described (see Section 3.3.6) in whom CSF PLP is reduced below the reference range. This finding could indicate another mechanism whereby central PLP deficiency develops.

Only the free vitamin bases can cross the blood-brain barrier, mainly at the choroid plexus; PLP is cleaved by tissue non-specific alkaline phosphatase to pyridoxal which is then actively transported first into the CSF and then by a similar mechanism into the brain cells. Once inside the brain cell, pyridoxal is rephosphorylated back into its active form by pyridoxal kinase and so is ‘trapped’ within the cell (29). When hypophosphatasia, PNPO and antiquitin deficiency have been excluded, it is feasible that a genetic disorder affecting transport mechanisms into the CNS could produce an elevated plasma: CSF PLP ratio with resulting epilepsy, however, at present, no such disorder has been described. Reduced activity of brain pyridoxal kinase offers an alternative explanation. The phenotype of pyridoxal kinase deficiency secondary to clock gene knockout has been demonstrated in animal models (41) where analysis of brain tissue demonstrates PLP, dopamine and serotonin deficiency and these mice die early due to a severe seizure disorder.

3.4.7 Nutrition

When metabolic pathways and transport systems are intact, plasma concentrations of PLP and thus nutritional status will impact upon CSF PLP concentration. Vitamin B₆ is ingested in the diet and as the vitamin is widespread in various food groups, isolated dietary deficiency in the general population is rare in developed countries (1). In children with seizure or movement disorders, however, nutrition may be inadequate for a variety of reasons including cessation of enteral feeding (with only provision of intravenous fluids), vomiting or poor gastrointestinal absorption. Simultaneous measurement of plasma PLP is vital to aid interpretation of CSF results however in our series this measurement was infrequently performed. Equally, information regarding nutritional intake was not available in many patients.
3.5 SUMMARY

In summary, this review has shown that previous, preliminary age related reference ranges for CSF PLP concentration are unsatisfactory for the patient groups in whom we are increasingly measuring it. A revised reference range with reduced lower reference limits is proposed, based upon four age groups.

It has been demonstrated that CSF PLP measurement is a useful part in the investigation of children with seizure and movement disorders and is best assessed in conjunction with other biochemical parameters particularly CSF monoamine metabolites. CSF PLP analysis is important for the diagnosis of PDE and PNPO deficiency and may aid diagnosis in MoCoF and sulphite oxidase deficiency and other disorders yet to be described. It can also play a useful role in monitoring treatment for example with L-dopa. It may also be informative to compare the concentration of PLP in CSF to the plasma PLP concentration; however measurement of plasma PLP alone is not diagnostic for vitamin B₆ related seizure disorders. Depletion of CSF PLP in parallel with 5-MTHF and BH₄ may be reflective of an inflammatory process in which these molecules can act as free radical scavengers. Other disorders that result in an increased production of reactive oxygen species (such as mitochondrial disorders) may produce a similar biochemical picture, with depletion of PLP and 5-MTHF.

By inactivation of PLP, use of sulphite containing drug preparations may increase the risk of seizures in vulnerable patient groups such as those who have a reduced seizure threshold due to their primary pathology or those who have other risk factors for reduced PLP. Seizures do not in themselves appear to influence CSF PLP levels and although this series did not demonstrate an association, it should be considered that some individual AED may theoretically reduce CSF PLP concentration.

Measurement of PLP as the sole B₆ vitamer in clinical practice is very useful but does have limitations. In order to further investigate those patients with reduced CSF PLP in whom a diagnosis has not been reached, use of advanced laboratory techniques to assess all B₆ vitameric forms will offer great advantages.
Chapter 4
Measurement of plasma B₆ vitamer profiles in children with epilepsy using an LC-MS/MS method
4.1 INTRODUCTION

Several inborn errors that affect the metabolic pathways of vitamin B$_6$ and cause epilepsy have been described as discussed in Section 1.1.13. They may be diagnosed by detecting elevated urinary α-aminoacid semialdehyde (α-AASA) in antiquitin deficiency (OMIM #266100) or by mutation analysis in pyridox(am)ine 5’ phosphate oxidase (PNPO) deficiency (OMIM #610090) and hypophosphatasia (OMIM 241500). Diagnosis may be supported by non-specific biochemical changes in cerebrospinal fluid PLP, neurotransmitter and amino acid profiles (113;327). However, whilst these are considered rare disorders, childhood seizures responsive to B$_6$ are not uncommon (125) and clinicians are increasingly aware of patients with seizures that respond empirically to pyridoxine or pyridoxal 5’-phosphate but do not fall into these recognised diagnostic groups. In order to explore the hypothesis that previously undescribed diseases may be caused by impaired interconversion of B$_6$ vitamers, laboratory methods are required to simultaneously measure the individual vitamers, pyridoxamine (PM), pyridoxal (PL), pyridoxine (PN), pyridoxal 5’-phosphate (PLP), pyridoxine 5’-phosphate (PNP) and pyridoxamine 5’-phosphate (PMP), and their breakdown product, 4-pyridoxic acid (PA).

Many children and adults are treated with large doses of vitamin B$_6$ for a variety of genetic (e.g. antiquitin deficiency and B$_6$ responsive homocystinuria) and non-genetic conditions (e.g. pyridoxine as an adjunct to isoniazid in tuberculosis treatment). Neurotoxicity associated with these mega doses of pyridoxine is well reported (93) but the underlying mechanisms are not well understood. Laboratory measurement of the various B$_6$ vitamers should allow for optimisation of treatment regimes, improve monitoring and may ultimately further understanding of B$_6$ toxicity.

4.1.1 Laboratory measurement of the B$_6$ vitamers

Historically indirect methods such as the tryptophan load have been used to assess vitamin B$_6$ status but these have obvious drawbacks, not least the burden upon the patient. Other examples include the use of the protozoan *Tetrahymena pyriformis* which utilises its dependence on B$_6$ for growth to provide an estimate of B6 in body fluids (328).
Such techniques have now been largely replaced by direct methods and one of the most commonly used direct laboratory techniques to measure the vitamers in plasma is high-pressure liquid chromatography (HPLC). Several variations of this methodology have been described in the literature however there are limitations with all; the majority do not measure all vitameric forms (329), some require columns that are no longer available (330;331) and some use cyanide derivatisation which, for safety reasons, is not ideal (332). In addition, as a general principle, HPLC technology may not offer sufficient sensitivity or specificity when measuring multiple analytes that are present in low concentrations in biological fluids (333).

Coupled to HPLC, use of mass spectrometry as the chromatographic detector (LC-MS) has clear advantages over HPLC technology alone and can overcome many of these problems; hence its use in routine diagnostic laboratories has increased in recent years. In particular LC-MS shows high specificity and is superior in its ability to analyse complex mixtures.

In principle, mass spectrometry is an analytical technique that measures the molecular mass of individual compounds and atoms very precisely by converting them into ions. It allows unsurpassed molecular specificity and provides ultrahigh detection sensitivity (334). Conversion of analyte molecules into gas–phase ionic species is the essential first step of the process allowing easy manipulation of the ion’s motion and direction. Various modes of ionisation are applied according to the nature of the sample and what information is required. Following this the molecular ions are separated and analysed on the basis of their mass-to-charge (m/z) ratio. In the final stage, the ion current generated by the separated ions is measured and displayed as a mass spectrum or an ion chromatogram by sophisticated software.

**4.1.2 Liquid chromatography mass spectrometry**

Many instruments are available that combine the various modes of sample provision, ionisation and ion analysis. Liquid chromatography-mass spectrometry, using electrospray ionisation and triple quadrupole mass analysers (LC-MS/MS) is a versatile technique used in clinical biochemistry laboratories and is the focus of this chapter.
4.1.3 High pressure liquid chromatography (HPLC)

This chromatography technique is used to separate compounds on the basis of time of elution from a stationary solid phase column and uses a liquid mobile phase forced onto the column at high pressures. Various aspects of the HPLC methodology ultimately influence its ability to separate compounds of a certain chemical nature, thus, method development involves optimisation of flow rates, column choice as well as the composition of the mobile phase component. Typical solvents used in HPLC such as methanol and acetonitrile are compatible with electrospay ionisation, however buffers containing phosphate or sodium acetate should be avoided as they will contaminate the source and create MS adducts.

4.1.4 Electrospray ionisation

Electrospray ionisation (ESI) is an ‘atmospheric pressure ionisation’ (API) technique that is particularly suitable for samples in liquid form. It is also very successfully coupled with a HPLC inlet method. It works well with polar molecules and thus is suited to the analysis of many metabolites and peptides.

In this form of ionisation, the liquid sample for analysis is pumped through a metal capillary maintained at 3 to 5 kV. It is then nebulised to produce a very fine spray of highly charged droplets within an intense electric field where evaporation of the solvent converts charged droplets into gas-phase ions. Evaporation is assisted by the flow of heated dry nitrogen gas. ESI can be performed in both a positive and negative ionisation mode and is controlled by selecting the capillary voltage bias, and this allows optimisation of sensitivity and specificity for a particular ion of interest. The ionised analytes are then focussed into the high vacuum of the mass spectrometer for analysis. As ESI imparts very little energy to the metabolite, minimal fragmentation occurs in the source and it is considered a ‘soft’ ionisation process (335;336).

This technique of ionisation is widely used for biological samples, however those molecules that are neutral or which have low polarity such as some lipids, are not efficiently ionised by ESI and alternatives such as atmospheric pressure chemical or photo-ionisation (APCI and APPI) are required.
4.1.5 Quadrupole analysers

The quadrupole analyser consists of four parallel metal rods to which a combination of constant and varying voltage is applied. This allows the transmission of a very narrow band of $m/z$ values along the rod axis and by varying the voltages with time, a wide range of $m/z$ values can be scanned, producing a mass spectrum (335).

Ions can be made to undergo fragmentation by collision with an inert gas such as argon in a process called ‘collision induced dissociation’ to produce a product or daughter ion. This may be achieved in a specifically designed quadrupole or collision cell and when placed between two quadrupole mass analysers, this is termed a triple quadrupole mass spectrometer (MS/MS). This two stage mass analysis has great advantages over single stage analysis due to its superior analysis specificity. For example, in a complex biological sample such as plasma it is likely that more than one component will produce an ion of a certain mass-to-charge ratio however, by adding a second stage of analysis there is only a low probability that they will both fragment in a similar manner to produce the same daughter or product ion. By using HPLC linked to MS/MS this specificity increases still further.

4.1.6 Considerations for the measurement of B$_6$ vitamers in plasma

Measurement of B$_6$ vitamers concentrations in plasma poses several potential problems, most of which can be overcome by LC-MS/MS analysis. For example, some of the vitamers are of a similar mass and chemical structure, they are all water soluble and highly polar molecules and in addition most are present at very low (nanomolar) concentrations in biological fluids.

One major challenge to measuring B$_6$ vitamers in plasma using LC-MS/MS is the effect of ion suppression. This term describes the reduction in signal observed when there is more than one component present in the ion source ‘competing’ in the ionisation process, as will be the case for human plasma. This can be improved to some extent in the sample preparation stage, for example by performing protein precipitation, but these procedures will not completely remove ion suppression effects. Because individual plasma samples will produce variable matrix effects (due
to differences in endogenous compounds) a stable isotope internal standard is required to prevent variable ion suppression affecting quantification (335).

The challenge of identifying compounds present at low concentrations in a complex mixture has been greatly advanced by the use of tandem mass spectrometry linked to HPLC and in this chapter an LC-MS/MS based method is presented to simultaneously measure all B₆ vitamers and pyridoxic acid in plasma. This method has been adapted from previous similar work (301), to enable its use on the instrumentation available in our laboratory. The method is applied to groups of children with inborn defects of B₆ metabolism, B₆ responsive seizures and other neurological disorders, focusing on the concentrations and interrelations of the B₆ forms in these disorders.

4.2 MATERIALS AND METHOD DEVELOPMENT

4.2.1 Chemical reagents

Pyridoxamine dihydrochloride, pyridoxal hydrochloride, pyridoxine hydrochloride, pyridoxamine 5’-phosphate, pyridoxal 5’-phosphate mono-hydrate and 4-pyridoxic acid were purchased from Sigma Aldrich (Gillingham, Dorset, UK).

Four deuterated vitamers were used as internal standards; Pyridoxal methyl d₃ hydrochloride [>98% atom %d] was purchased from Isotec, pyridoxine d₂ hydrochloride (5-hydroxymethyl-d2) [>98% atom %d] from CDN Isotopes, 4-pyridoxic acid d₅ (5,5’-d2) [>98%] was purchased from Buchem BV and d₂ pyridoxal 5’-phosphate was kindly supplied as a gift by Professor Coburn, Department of Chemistry, Indiana University, Purdue University, Forte Wayne.

HPLC grade methanol was purchased from Fisher Scientific. Acetic acid, heptafluorobutyric acid (HFBA) and trichloroacetic acid (TCA) were purchased from Sigma Aldrich.

Stock solutions of B₆ vitamers, pyridoxic acid and the deuterated internal standards were made with purified deionised water and stored at -80°C. They were placed on ice and protected from the light during laboratory handling.
All vitamer calibrators were checked for the presence of other B₆ vitamers and deuterated internal standards were analysed to check for the presence of the non-deuterated species. No appreciable amounts were detected using the method described in this paper, making them suitable for use in method development. Examples of this for the deuterated compounds d2PLP and d3PL are shown in Figure 22.
Figure 22. Chromatogram of deuterated pyridoxal (d3PL) in (A) and deuterated pyridoxal 5'-phosphate (d2PLP) in (B) illustrating that neither compound is contaminated by the measured other B₆ vitamers measured. Precursor and product ion are shown for each vitamer investigated.
4.2.2 Sample collection & preparation

Pooled plasma for calibration, recovery and imprecision studies was collected from healthy adults not taking any medications or vitamin supplementation. Clinical plasma samples for the work presented here were obtained with consent under two ethically approved projects from patients attending Great Ormond Street Hospital for Children, London (REC numbers 09/H0706/85 and 04/Q0508/81).

Venous blood samples were taken into EDTA tubes (Sarstedt Monovette®) and were centrifuged at 7,000 x g for 10 minutes at 4 °C (accuSpin; Micro R. Fisher Scientific) within 60 minutes of collection and the plasma removed immediately. Plasma samples were then stored at -80 °C until further use. At the time of analysis, plasma samples were defrosted on ice and protected from light. Proteins were precipitated by mixing 60 μL of plasma with an equal volume of 0.3 N TCA (containing deuterated internal standards) to a final TCA concentration of 0.15 N. The sample was vortexed thoroughly for 30 seconds, left on ice in the dark for 60 minutes and finally centrifuged at 7,000 x g for 10 minutes at 4 °C. The resulting supernatant was transferred to a HPLC vial and placed in an autosampler where the samples were kept at 4 °C and protected from light. Previous work has shown B₆ vitamers to be stable under these conditions (301).

4.2.3 High Pressure Liquid chromatography

A Waters Alliance 2795 LC system was used for delivery of the mobile phase.

4.2.3.1 HPLC column selection

A HS F5 column (Supelco; 10 cm x 2.1 mm; 3 μm) fitted with a HS F5 guard column (2 cm x 2.1 mm; 5 μm) was used for analysis. This column consists of a pentafluorophenylpropyl bonded phase. The five fluorine groups of this stationary phase have a strong electron drawing effect and it has both reversed phase and Hydrophilic Interaction Chromatography (HILIC) modes. It was chosen because of its properties to separate small, polar, water soluble molecules and because it is tolerant of a low pH.
Due to the instrument parameters, a maximum mobile phase flow rate of 0.2 ml per minute was used which was achieved with maximum column pressures of 2000 psi. The column and guard were kept at a constant temperature of 30 °C during the analysis.

4.2.3.2 Optimisation of the mobile phase

For successful analysis of B₆ vitamers in plasma the mobile phase composition proved to be very important.

During the initial experiments when aqueous B₆ standards were used, it was only found necessary to use two components in the mobile phase; 100% methanol and 3.7% acetic acid. In the subsequent work using human plasma however it proved very difficult to achieve adequate sensitivity to detect the phosphorylated compounds (PLP and PMP) using only these two solvents. It was considered that in order for PLP and PMP to be retained better on the column and thus improve sensitivity an ion pairing agent may be required.

Heptafluorobutyric acid (HFBA) which is a volatile perfluorinated carboxylic acid was the selected ion pairing agent and experiments were designed to assess its effect at varying concentrations on the measurement of vitamers in plasma. The addition of HFBA at 100 mM concentration in 3.7% acetic acid present continuously (as 2.5% of the mobile phase composition) showed the most beneficial effect on the detection of PLP and PMP. As shown in Figures 23 & 24, sensitivity was increased for PMP and peak shapes were improved for both PLP and PMP.

HFBA affected some of the other vitamers in a detrimental manner. Sensitivity for PN and PL was reduced however it remained more than adequate for analysis and was acceptable given the improvement in phosphorylated species.

HFBA also affected the retention times of many vitamers. The retention times of the following vitamers were increased (by the amount of time shown in brackets) PMP (0.3 min); PL (3.0 min); PN (3.5 min); PM (9.0 min) and that of PA was decreased (0.6 min). This is also illustrated in Figure 24.
Figure 23. Chromatogram of B₆ vitamers in control plasma spiked with 100 nmol/L calibrator vitamer standards [40 μL injection using mobile phase consisting of methanol and 3.7% acetic acid]
Figure 24. Chromatogram of B6 vitamers in control plasma spiked with 100 nmol/L calibrator vitamer standards showing improved sensitivity and peak shape for PLP and PMP with addition of HFBA to mobile phase [40 μL injection using a mobile phase consisting of methanol; 3.7% acetic and 100 mM HFBA in 3.7% acetic acid]
The final mobile phase consisted of the following three components: (A) 100% methanol; (B) 3.7% acetic acid; (C) 3.7% acetic acid containing 100 mM HFBA. This acidic mobile phase with an ion pairing agent is suited to the detection of highly polarised compounds such as B₆ vitamers. The best chromatographic separation and peak shapes were achieved with the gradient profile shown in Table 12. Each gradient step was linear.

<table>
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<th>Time (minutes)</th>
<th>100% methanol (%)</th>
<th>3.7% acetic acid (%)</th>
<th>3.7% acetic acid &amp; 100 mM HFBA (%)</th>
<th>Flow rate (ml/min)</th>
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<td>0.2</td>
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</tbody>
</table>

### 4.2.4 Electrospray ionisation-tandem mass spectrometry (ESI-MS/MS)

Mass spectrometry of B₆ vitamers and PA was carried out using a triple quadrupole Micro Quattro instrument (MicroMass, Waters, UK) fitted with an electrospray ionisation source. The source and desolvation gas temperature were held constant at 150 °C and 350 °C respectively, with flow rates of 950 and 60 litres of nitrogen per hour.

B₆ vitamers and PA were detected using multiple reaction monitoring mode (MRM) and the settings optimised by infusion of each vitamer in proportions of mobile phase according to time of elution off the HPLC column. The mass spectrometer was
operated in the positive ion mode for analysis of all B<sub>6</sub> vitamers and PA. A single scan segment of 25 minutes provided adequate sensitivity for detection and measurement of all species, giving a total sample run time of 25 minutes which included time for column re-equilibration. The final chosen parameters for the parent and most abundant daughter ion are as detailed in Table 13.

Parent-daughter ion pairs suggested a loss of H<sub>2</sub>O (18) for PL; loss of ammonia (17) and H<sub>2</sub>O (18) for PM; loss of 2.H<sub>2</sub>O (36) for PN; loss of HPO<sub>3</sub> and water (98) for PLP; loss of H<sub>2</sub>O (18) HPO<sub>3</sub> (98) and ammonia (17) for PMP and loss of 2.H<sub>2</sub>O (36) for PA. As PNP was not available as a calibration vitamer, an additional transition was added to the MS file for this compound using a theoretical fragmentation pattern due to loss of phosphate and water (116) (based on the fragmentation of PLP and PN).

All compounds could be differentiated on the basis of both m/z ratio transitions and retention times. PLP and PMP had very similar retention times and parent ions that differed by only 1 Da. There was, however, no cross talk between ion pairs originating from these two different analytes (Figure 25).
Table 13. The mass spectral specifications for detection of B₆ vitamers in plasma and their deuterated internal standards

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (minutes)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>9.30</td>
<td>168.2</td>
<td>150.0</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>16.10</td>
<td>169.2</td>
<td>133.9</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>11.10</td>
<td>170.2</td>
<td>134.0</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Pyridoxic Acid</td>
<td>7.60</td>
<td>184.2</td>
<td>148.0</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>3.94</td>
<td>248.1</td>
<td>150.0</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Pyridoxamine phosphate</td>
<td>3.89</td>
<td>249.1</td>
<td>134.0</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Pyridoxine phosphate</td>
<td>3.86</td>
<td>250.1</td>
<td>134.0</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>d3 pyridoxal</td>
<td>9.30</td>
<td>171.2</td>
<td>153.0</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>d2 pyridoxine</td>
<td>11.10</td>
<td>172.2</td>
<td>136.0</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>d2 pyridoxic acid</td>
<td>7.60</td>
<td>186.2</td>
<td>150.0</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>d2 pyridoxal phosphate</td>
<td>3.90</td>
<td>250.1</td>
<td>152.0</td>
<td>27</td>
<td>19</td>
</tr>
</tbody>
</table>
Figure 25. The chromatogram of aqueous PLP (A) and PMP (B) standards to illustrate that despite similar retention times and parent ion mass there is no cross talk between channels.

During the process of method development it was observed that the sensitivity for PLP was paradoxically improved with a smaller injection volume and a similar effect was seen for PL. As may be expected the other vitamers were better detected at larger injection volumes, although this improvement was only modest for PMP. As it was vital that the method provided good sensitivity for PLP, an injection volume of 25 μL was chosen which allowed good detection of PLP with an acceptable sacrifice in sensitivity for the other vitamers (Table 14).
Table 14. Variation in signal intensity with injection volume for the B₆ vitamers measured in control plasma spiked with 50 nmol/L of calibrator vitamers

<table>
<thead>
<tr>
<th>Injection volume</th>
<th>20 μL</th>
<th>25 μL</th>
<th>30 μL</th>
<th>40 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>1100</td>
<td>1195</td>
<td>900</td>
<td>640</td>
</tr>
<tr>
<td>PMP</td>
<td>279</td>
<td>456</td>
<td>483</td>
<td>605</td>
</tr>
<tr>
<td>PA</td>
<td>1596</td>
<td>2736</td>
<td>3602</td>
<td>6236</td>
</tr>
<tr>
<td>PN</td>
<td>8254</td>
<td>10085</td>
<td>11808</td>
<td>13750</td>
</tr>
<tr>
<td>PL</td>
<td>1867</td>
<td>1627</td>
<td>1077</td>
<td>1101</td>
</tr>
<tr>
<td>PM</td>
<td>4937</td>
<td>5479</td>
<td>5768</td>
<td>10013</td>
</tr>
</tbody>
</table>

[Mobile phase containing methanol, 3.7% acetic acid and 100 mM HFBA as per Table 12]

In the final method a 25 μL volume of deproteinised plasma was injected every 25 minutes; the column effluent was then delivered to the mass spectrometer with the first 1 minute and last 5 minutes being diverted to waste. MassLynx software was used for data acquisition and analysis.

4.2.4.1 Quantification of B₆ vitamers and pyridoxic acid

B₆ vitamers and PA were quantified in plasma by the addition of a known concentration (100 nM) of deuterated internal standard. The same concentration of internal standard was used to construct calibration curves with reference vitamers (except PNP which was not available) and PA. The amount of PLP and PMP present was calculated from the ratio of the signal area for the vitamer to the signal area for d₂ PLP. Similarly d₃ PL was used to calculate the amount of PL and PM present, d₂ PN for PN and d₂ PA for PA. PNP was quantitated by determining the ratio of the signal from PNP to the signal from deuterated PLP. This was converted to a concentration in plasma by using the calibration curve for PLP. If the calibration for PNP was identical to that for PLP this would be the plasma concentration of PNP in nmol/L, however we cannot make that assumption and so PNP concentrations are expressed in the results as “concentration units”.

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4.3 RESULTS

4.3.1 Calibration curves and linearity

To estimate the linear range of the method, calibration curves in the range 1 – 500 nmol/L were constructed for each vitamer in water and plasma (Figures 26 & 27). This range was chosen because it covers the physiological range expected to be encountered in the analysis of human plasma samples. Values for $r^2$ of equal to or greater than 0.98 were achieved for each vitamer by linear regression (Table 15 & 16).
Figure 26. Calibration curves of B₆ vitamers in water
Figure 27. Calibration curves of B₆ vitamers in plasma
Table 15. Linearity of B₆ vitamers in water over a physiological range (1 – 500 nmol/L)

<table>
<thead>
<tr>
<th>Regression parameters</th>
<th>Slope</th>
<th>X-intercept</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>0.019</td>
<td>-2.6</td>
<td>0.99</td>
</tr>
<tr>
<td>PMP</td>
<td>0.008</td>
<td>-8.6</td>
<td>0.99</td>
</tr>
<tr>
<td>PA</td>
<td>0.027</td>
<td>5.14</td>
<td>0.99</td>
</tr>
<tr>
<td>PN</td>
<td>0.015</td>
<td>-1.5</td>
<td>0.98</td>
</tr>
<tr>
<td>PM</td>
<td>0.012</td>
<td>3.0</td>
<td>0.99</td>
</tr>
<tr>
<td>PL</td>
<td>0.025</td>
<td>5.9</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 16. Linearity of B₆ vitamers in plasma over a physiological range (1 – 500 nmol/L)

<table>
<thead>
<tr>
<th>Regression parameters</th>
<th>Slope</th>
<th>X-intercept</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>0.019</td>
<td>-145</td>
<td>0.99</td>
</tr>
<tr>
<td>PMP</td>
<td>0.008</td>
<td>-16.8</td>
<td>0.99</td>
</tr>
<tr>
<td>PA</td>
<td>0.025</td>
<td>-61.4</td>
<td>0.99</td>
</tr>
<tr>
<td>PN</td>
<td>0.015</td>
<td>2.08</td>
<td>0.99</td>
</tr>
<tr>
<td>PM</td>
<td>0.014</td>
<td>0.12</td>
<td>0.99</td>
</tr>
<tr>
<td>PL</td>
<td>0.027</td>
<td>-48.4</td>
<td>0.99</td>
</tr>
</tbody>
</table>
4.3.2 Precision studies: Intra- and inter-batch coefficient of variance and recovery studies

Assay precision was assessed by calculation of the intrabatch and interbatch coefficient of variance (CV) \[ CV\% = \frac{\text{standard deviation}}{\text{mean}} \times 100 \] in plasma for each vitamer and by recovery of each analyte \[ \text{recovery}\% = \frac{\text{measured concentration} - \text{endogenous concentration}}{\text{concentration added}} \times 100 \]

Plasma was spiked with medium (50 nmol/L) and high (100 nmol/L) concentrations of each vitamer. One batch was left ‘unspiked’ with endogenous concentrations of B₆ vitamers.

10 samples with each added concentration [50 nmol/L (medium) and 100 nmol/L (high)] were analysed for calculation of the intrabatch CV. The intrabatch CV for each vitamer was <10% (Table 17).

<p>| Table 17. Intra- and interbatch co-efficient of variation (CV) and recovery for B₆ vitamers in plasma |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Intrabatch CV (%) (n=10)</th>
<th>Interbatch CV (%) (n=5)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>high</td>
<td>medium</td>
</tr>
<tr>
<td>PLP</td>
<td>2.6</td>
<td>5.3</td>
</tr>
<tr>
<td>PMP</td>
<td>9.5</td>
<td>8.8</td>
</tr>
<tr>
<td>PA</td>
<td>5.3</td>
<td>4.3</td>
</tr>
<tr>
<td>PN</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>PM</td>
<td>8.7</td>
<td>3.8</td>
</tr>
<tr>
<td>PL</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Interbatch CV’s were calculated using one sample run in triplicate with the two different added concentrations (50 nmol/L and 100 nmol/L) on five different days (not including the samples used to calculate intrabatch CV) over a period of 4 weeks. CV’s for PLP, PA, PN and PL were <10% but the CV for PM and PMP were higher at 17.0% and 17.8% respectively (Table 17). This was felt to be acceptable for use in clinical samples where PM and PMP are not expected to be present.

The intrabatch CVs reported here are better than those reported by Midttun et al. (301) however the interbatch CVs are comparable.

Recovery of B₆ vitamers from plasma ranged from 68-124% (Table 17). For the three compounds always present and for which accurate measurement is most important (PLP, PL and PA) recoveries were 88 – 116%. Recoveries are similar to those reported by Midttun et al. (301).

4.3.3 Indicators of accuracy

PLP of a known concentration in plasma is available from Chromsystems® (Munich, Germany) for use as a quality control standard in their HPLC B₆ analysis kit. This method is described in Section 2.2.1. Analysis of this standard in duplicate on three occasions using the LC-MS/MS method produced comparable results to those of the Chromsystems method (LC-MS/MS method 70; 72; 98 nmol/L; Chromsystems method 68; 67; 86 nmol/L). In addition 39 plasma samples were run in parallel on both systems to directly compare the two methods which showed good agreement; \( r^2 = 0.95, p<0.0001 \) (Figure 28).
Figure 28. Comparison of plasma PLP measured by HPLC and LC-MS/MS method

4.3.4 Study of matrix effects

The possible matrix effects of plasma on detection of B₆ vitamers were studied by measurement of the peak intensities for each vitamer in plasma spiked with differing concentrations of vitamers (13 concentrations, range 3 – 500 nmol/L). These intensities were compared to peak intensities for the vitamers in water where matrix effect % = [(peak area spiked – peak area endogenous)/peak area in water] x100.

Effects of ion suppression were seen for all vitamers in plasma as follows; PL 33%; PM 69%; PN 71%; PA 79%; PMP 86% and PLP 96%. While these figures (particularly those for PL, PM and PN) are lower than for an ideal method, the recovery experiments indicated that the internal standards were adequate to compensate for the matrix effects.

4.3.5 Stability of standards and plasma samples

Vitamin B₆ is light and temperature sensitive. The aqueous B₆ standards and spiked pooled plasma samples were handled as detailed above and stored in aliquots at
were demonstrated to be stable over a 6 month period by repeated measurement of the same samples.

4.3.6 Application of the analytical method to paediatric populations including children with inborn errors of vitamin B₆ metabolism

The LC-MS/MS method was used to measure B₆ vitamer and PA profiles in children with inborn errors of vitamin B₆ metabolism and in children in whom a definitive diagnosis had not been made but who had a B₆ responsive seizure disorder. It should be noted that many of these patients were on treatment doses of vitamin B₆ (pyridoxine hydrochloride 200 mg per day or pyridoxal 5’-phosphate 30 mg/kg/d) at the time of sampling (Table 18-21). In order to establish a reference range, control plasma samples were collected from paediatric outpatient clinics. No control patient was using vitamin supplementation or dietary manipulation of any form at the time of sampling. Some were taking medications but these were not considered to have an important effect on B₆ metabolism.

Each patient sample was analysed in duplicate and the mean of each is presented in Tables 18-21. B₆ vitamers expressed as ratios; PLP:PL and PL:PA, are also presented in these tables.

4.3.6.1 PNPO deficiency

Table 18 shows the profile of B₆ vitamers from three patients (1, 2 & 3) with genetically proven PNPO deficiency on treatment with PLP (30 mg/kg/d in Patient 1 & 2) and PN (Patient 3). The chromatogram for Patient 1 is shown in Figure 29. This distinct pattern demonstrates significantly elevated PLP, PL and PA and agrees with published data for individuals on B₆ supplementation (301;337). In addition, as may be expected in the case of severely reduced PNPO activity, the two individuals on PLP therapy (Patients 1 & 2) have significantly elevated PM, PMP, PN and PNP compared to age matched controls and to patients with other inborn errors of B₆ metabolism on B₆ supplementation. The child on PN therapy (Patient 3) does not exhibit elevated PNP or PMP, however PN and PM are substantially elevated.
4.3.6.2 Antiquitin deficiency

Plasma B₆ levels were measured in four patients from two families with antiquitin deficiency, all were on PN therapy. Patients 4 and 5 (siblings) have levels of PLP in plasma that are below the reference range, with a markedly reduced PLP: PL ratio. PL and PA are strongly elevated in keeping with increased catabolism of PN and PLP through intact pathways. PM, PMP and PN are modestly elevated; PNP is not detectable. Patients 6 & 7 (twins) have a different profile to patients 4 & 5 showing a markedly elevated PLP with PL and PA elevated but not to such high levels. None of the other vitamers are elevated in these two patients.
4.3.6.3 Molybdenum Cofactor (MoCoF) deficiency

Patients 8 and 9 (Table 18) with genetically proven MoCoF deficiency have reduced or absent activity of the MoCoF dependent enzymes sulphite, xanthine and aldehyde oxidase (AOX). AOX catalyses the formation of PA from PL for excretion in urine and evidence of reduced activity of this enzyme seen in these patients is demonstrated by an elevated PL to PA ratio and a reduced absolute PA concentration (Table 18).
Table 18. Plasma B₆ vitamer profiles in patients with an inborn error affecting vitamin B₆ metabolism

<table>
<thead>
<tr>
<th>Diagnosis and clinical information</th>
<th>Age (documented where known)</th>
<th>Medication**</th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
<th>PN</th>
<th>PNP</th>
<th>PMP</th>
<th>PM</th>
<th>PLP:PL</th>
<th>PL:PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference range n=24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.3y – 16y</td>
<td></td>
<td>46-321</td>
<td>4.6-18.1</td>
<td>16.4-139</td>
<td>nd-0.62</td>
<td>nd</td>
<td>nd-9.3</td>
<td>nd</td>
<td>5.2 – 18.6</td>
<td>0.1 – 0.7</td>
</tr>
<tr>
<td>1 PNPO</td>
<td>2y 2m</td>
<td>PLP [30 mg/kg/d]</td>
<td>580</td>
<td>426.8</td>
<td>792.8</td>
<td>575</td>
<td>43</td>
<td>18</td>
<td>192.7</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td>2 PNPO</td>
<td>10y 2m</td>
<td>PLP [30 mg/kg/d]</td>
<td>632.6</td>
<td>5798</td>
<td>7926.3</td>
<td>598.8</td>
<td>77.17</td>
<td>101</td>
<td>2731</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>3 PNPO</td>
<td>8y 1m</td>
<td>PN</td>
<td>839.8</td>
<td>4974.4</td>
<td>4327.7</td>
<td>974.9</td>
<td>nd</td>
<td>nd</td>
<td>135.4</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>4* PDE</td>
<td>10y 5m</td>
<td>PN [100 mg BD]</td>
<td>23.9</td>
<td>6351.4</td>
<td>5903.7</td>
<td>60.4</td>
<td>nd</td>
<td>14.9</td>
<td>107.6</td>
<td>0.0038</td>
<td>1.1</td>
</tr>
<tr>
<td>5* PDE</td>
<td>12y 2m</td>
<td>PN [100 mg BD]</td>
<td>11.4</td>
<td>6475.8</td>
<td>5068.8</td>
<td>7.2</td>
<td>nd</td>
<td>23.57</td>
<td>143.8</td>
<td>0.0018</td>
<td>1.3</td>
</tr>
<tr>
<td>6 PDE</td>
<td>8y 7m</td>
<td>PN [100 mg BD]</td>
<td>587.9</td>
<td>198.4</td>
<td>238.8</td>
<td>0.35</td>
<td>nd</td>
<td>nd</td>
<td>3.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>7 PDE</td>
<td>8y 7m</td>
<td>PN [100 mg BD]</td>
<td>603.3</td>
<td>202.6</td>
<td>320.9</td>
<td>0.37</td>
<td>nd</td>
<td>nd</td>
<td>3.0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MoCoF</td>
<td>17y 11m</td>
<td>Nil</td>
<td>Phenobarbitone, baclofen, L-Dopa</td>
<td>153.6</td>
<td>19</td>
<td>10.5</td>
<td>0.5</td>
<td>6.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>---------</td>
<td>-----</td>
<td>---------------------------------</td>
<td>-------</td>
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<td>------</td>
<td>-----</td>
<td>-----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>9</td>
<td>MoCoF</td>
<td>11m</td>
<td></td>
<td></td>
<td>235.3</td>
<td>140.3</td>
<td>7.7</td>
<td>0.4</td>
<td>nd</td>
<td>6.1</td>
<td>nd</td>
</tr>
</tbody>
</table>

All units nmol/L, except PNP which is expressed as ‘concentration units’. Values outside reference range are shown in bold. nd = not detected; BD = twice per day; y = years; m = months; n = number of samples analysed

* peripheral neuropathy on B₆ supplementation; ** control patients were taking the following medication: simvastatin (n=3); carnitine (n=1); amlodipine (n=1); trimethoprim (n=1)
Figure 29. Chromatogram of B₆ vitamers in a patient with PNPO deficiency on PLP treatment
4.3.6.4 B₆ responsive seizure disorders (not Antiquitin or PNPO deficiency)

Patients 10 to 15 (Table 19 & 20) have a seizure disorder that is fully or partially responsive to PN or PLP, but do not have elevated urinary α-AASA (i.e. antiquitin deficiency) or mutations in PNPO. They had normal alkaline phosphatase levels for age (which excludes hyper- and hypophosphatasia) and did not exhibit the clinical features seen in hyperphosphatasia, or hypophosphatasia. In addition they were not taking medications known to affect vitamin B₆ metabolism.

Patient 11(a) (Table 19) and 14(a) (Table 20) have a profile similar to that of controls and, on B₆ supplementation, all patients have elevated levels of PLP, PL and PA. Compared to the reference population, a modest elevation of both PN and PM is seen in patient 11(b) (Table 19).
Table 19. Plasma B₆ vitamer profiles in paediatric patients with a seizure disorder fully responsive to vitamin B₆ (PNPO and α-AASA negative)

<table>
<thead>
<tr>
<th>Diagnosis and clinical information</th>
<th>Age</th>
<th>Medication**</th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
<th>PN</th>
<th>PNP</th>
<th>PMP</th>
<th>PM</th>
<th>PLP:PL</th>
<th>PL:PA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference range n=24</strong></td>
<td>4.3y – 16y</td>
<td>(dose documented where known)</td>
<td>46-321</td>
<td>4.6-18.1</td>
<td>16.4-139</td>
<td>nd-0.62</td>
<td>nd</td>
<td>nd-9.3</td>
<td>nd</td>
<td>5.2 – 18.6</td>
<td>0.1 – 0.7</td>
</tr>
<tr>
<td>10 PN responsive</td>
<td>3y 7m</td>
<td>PN [100 mg BD]</td>
<td>877</td>
<td>7385.8</td>
<td>6510</td>
<td>5.1</td>
<td>5.9</td>
<td>5.7</td>
<td>2.2</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>11 PLP responsive</td>
<td>(a) 5y 2m</td>
<td>AED</td>
<td>75.2</td>
<td>6.8</td>
<td>20.6</td>
<td>nd</td>
<td>3.9</td>
<td>9.3</td>
<td>nd</td>
<td>11.1</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(b) 6y 5m</td>
<td>AED and PLP [30 mg/kg/d]</td>
<td>709.4</td>
<td>7893</td>
<td>7371</td>
<td>32</td>
<td>nd</td>
<td>nd</td>
<td>28</td>
<td>0.09</td>
<td>1.1</td>
</tr>
<tr>
<td>12 PLP responsive</td>
<td>4y 1m</td>
<td>PLP</td>
<td>630.9</td>
<td>654.7</td>
<td>494.9</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>13 PLP responsive</td>
<td>1y 4m</td>
<td>PLP [30 mg/kg/d]</td>
<td>755.6</td>
<td>1102.7</td>
<td>3431.6</td>
<td>1.6</td>
<td>11.5</td>
<td>11.0</td>
<td>nd</td>
<td>0.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>

All units nmol/L, except PNP which is expressed as ‘concentration units’. Values outside reference range are shown in bold. nd = not detected; BD = twice per day; AED = antiepileptic drug, unspecified; y = years; m = months; n = number of samples analysed ** control patients were taking the following medication: simvastatin (n=3); carnitine (n=1); amlodipine (n=1); trimethoprim (n=1)
Table 20. Plasma $B_6$ vitamer profiles in paediatric patients with a seizure disorder partially responsive to vitamin $B_6$ (PNPO and $\alpha$-AASA negative)

<table>
<thead>
<tr>
<th>Diagnosis and clinical information</th>
<th>Age</th>
<th>Medication**</th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
<th>PN</th>
<th>PNP</th>
<th>PMP</th>
<th>PM</th>
<th>PL:PL</th>
<th>PL:PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference range n=24</td>
<td>4.3y – 16y</td>
<td>(dose documented where known)</td>
<td>46-321</td>
<td>4.6-18.1</td>
<td>16.4-139</td>
<td>nd-0.62</td>
<td>nd</td>
<td>nd-9.3</td>
<td>nd</td>
<td>5.2 – 18.6</td>
<td>0.1 – 0.7</td>
</tr>
<tr>
<td>14 Asperger syndrome and seizures. PN and PLP responsive</td>
<td>(a) 11y11m</td>
<td>AED</td>
<td>54.5</td>
<td>6.4</td>
<td>38.6</td>
<td>0.5</td>
<td>nd</td>
<td>5.13</td>
<td>nd</td>
<td>8.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(b) 12y 8m</td>
<td>PN [100 mg BD]</td>
<td>528.8</td>
<td>8532.2</td>
<td>5212.4</td>
<td>1.9</td>
<td>nd</td>
<td>4.6</td>
<td>3.1</td>
<td>0.1</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>(c) 13y 3m</td>
<td>PLP [30 mg/kg/d]</td>
<td>306.4</td>
<td>8452.5</td>
<td>5031.9</td>
<td>2.7</td>
<td>nd</td>
<td>4.1</td>
<td>5.1</td>
<td>0.04</td>
<td>1.7</td>
</tr>
<tr>
<td>15 Partially PLP responsive seizures</td>
<td>2y 7m</td>
<td>PLP [30 mg/kg/d]</td>
<td>478.4</td>
<td>102.4</td>
<td>144.6</td>
<td>0.31</td>
<td>nd</td>
<td>6.8</td>
<td>nd</td>
<td>4.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

All units nmol/L, except PNP which is expressed as ‘concentration units’. Values outside reference range are shown in bold. nd = not detected; BD = twice per day; AED = antiepileptic drug, unspecified; y = years; m = months; n = number of samples analysed ** control patients were taking the following medication: simvastatin (n=3); carnitine (n=1); amlodipine (n=1); trimethoprim (n=1)
4.3.6.5 Other patients

A pregnant adult female (Patient 16) (Table 21) had a reduced concentration of plasma PLP and PA and elevated PLP: PL ratio. Patient 17 (Table 21) on treatment with L-Dopa for an idiopathic dystonic movement disorder had a reduced plasma PLP and corresponding PL and PA. The plasma B₆ vitamer profile was measured in Patient 18 (Table 21) at an age that falls outside of the reference range quoted. He had a disproportionately elevated plasma PL as evidenced by increased PL: PA ratio.
Table 21. Plasma B₆ vitamer profiles in other patients

<table>
<thead>
<tr>
<th>Diagnosis and clinical information</th>
<th>Age</th>
<th>Medication**</th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
<th>PN</th>
<th>PNP</th>
<th>PMP</th>
<th>PM</th>
<th>PLP:PL</th>
<th>PL:PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference range n=24</td>
<td>4.3y – 16y (dose documented where known)</td>
<td>46-321</td>
<td>4.6-18.1</td>
<td>16.4-139</td>
<td>nd-0.62</td>
<td>nd</td>
<td>nd-9.3</td>
<td>nd</td>
<td>5.2 – 18.6</td>
<td>0.1 – 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Pregnancy (3rd trimester)</td>
<td>19y 5m</td>
<td>Nil</td>
<td>11.1</td>
<td>4.6</td>
<td>14.4</td>
<td>0.3</td>
<td>nd</td>
<td>2.3</td>
<td>nd</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>17 Idiopathic dystonia, speech and language disorder</td>
<td>5y 5m</td>
<td>Carbidopa</td>
<td>21.9</td>
<td>5</td>
<td>12.4</td>
<td>0.3</td>
<td>2.6</td>
<td>nd</td>
<td>nd</td>
<td>4.4</td>
<td>0.4</td>
</tr>
<tr>
<td>18 Epileptic encephalopathy, mitochondrial respiratory chain phenotype</td>
<td>4m</td>
<td>AED</td>
<td>170.6</td>
<td>40.2</td>
<td>42.7</td>
<td>0.3</td>
<td>5.8</td>
<td>nd</td>
<td>nd</td>
<td>4.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

All units nmol/L, except PNP which is expressed as ‘concentration units’. Values outside reference range are shown in bold. nd = not detected; BD = twice per day; AED = antiepileptic drug, unspecified; y = years; m = months; n = number of samples analysed; ** control patients were taking the following medications: simvastatin (n=3); carnitine (n=1); amlodipine (n=1); trimethoprim
4.4 DISCUSSION

Vitamin B₆ responsive seizures are an important cause of childhood epilepsy with up to 15% of idiopathic epilepsy responding to treatment with PN or PLP (125). Some of these children have a genetic diagnosis but many do not. New laboratory techniques are required to advance understanding in this field as the majority of established methods used for the determination of vitamin B₆ in biological samples are not capable of measuring all of the individual B₆ forms or do not have adequate sensitivity and specificity.

This chapter describes an LC-MS/MS method to measure B₆ vitamer profiles in human plasma and its application to (predominantly) paediatric patient populations. Performance of the method has been validated with regard to linearity over a physiological range, precision, and recovery and it is shown to be sufficiently sensitive to detect vitamer concentrations in the low nanomolar range. It has the advantage of using small sample volumes with a quick throughput time which can easily be applied for use in patient populations.

4.4.1 Plasma B₆ vitamer profiles in the reference population and those taking B₆ supplementation

The method was used to measure plasma B₆ concentrations in children with genetically proven inborn errors of B₆ metabolism, those without a diagnosis who have B₆ responsive seizures and also in a reference paediatric population (Tables 18-21). The reference population samples were drawn from outpatient clinics and covered an age range from early to late childhood. It did not include neonatal subjects or children in infancy, but provides a suitable range for most patients described in this study. Plasma B₆ concentrations in this reference population were comparable to those reported previously (301;337). Few healthy children are on high dose B₆ supplementation hence a reference range for this group of children is difficult to establish but should be pursued. At present comparisons to adult populations taking a B₆ supplement from the literature are made.
Previous work has shown that individuals on modest pyridoxine supplementation (40 mg/day) have elevated concentrations of PLP, PL and PA in plasma. PLP does not rise in a dose dependent manner, but increases to a threshold beyond which it does not increase. It is known that PLP is released from the liver into the circulation bound primarily to the lysine-190 residue of albumin and this protein-binding of PLP is believed to protect the coenzyme from hydrolysis and other reactions. The threshold seen for PLP in the case of supplementation possibly reflects the binding capacity of albumin. PL and PA however rise proportionally with dose as the flux through the catabolic pathway is increased (301;337). In these previous studies PN was detectable in the plasma of approximately half of the individuals taking PN supplementation but in the others it was ‘undetectable’ (301). PMP was not detectable in any samples and PM only in three individuals at low concentrations. PNP detected in the plasma samples of patients taking both placebo and PN supplementation by Midttun et al. was attributed by them to sample haemolysis in their series, although significant haemolysis is likely to result in elevated PMP in addition to PNP.

In the current study, despite being on treatment doses of B₆ (Table 18), the plasma vitamer profiles appear to show a characteristic pattern in some disease groups. This has not been reported previously and may be of use in clinical practice to aid diagnosis alongside existing methods. Measurement in much larger patient groups is necessary, however, before we can be confident of using a B₆ profile alone for diagnosis. Future work should also examine profiles in untreated patient groups although in practice, where treatment with B₆ is often urgent, these samples may be difficult to collect.

4.4.2 PNPO deficiency and other PLP responsive patients

In two children (Patient 1 & 2, Table 18) with PNPO deficiency on PLP treatment, reduced enzyme activity is evidenced by the accumulation of PM, PMP, PN and PNP. The presence of PMP and PM highlights the recycling role of this enzyme in normal human metabolism where PMP is recycled via a salvage pathway back to PLP (see Section 1.1.4.1). By contrast, the source of PN and PNP is likely to be attributable to the diet or multivitamin supplement as man is not capable of synthesising PN or PNP de novo.
Patient 3 has PNPO deficiency with epilepsy that responds to pyridoxine. He is part of a newly emerging PNPO phenotype that, in contrast to the originally described patients in whom PN treatment was ineffective, responds well to PN and does not require PLP treatment (unpublished observation). Given our current understanding of the enzymatic pathway for B₆ vitamer interconversion, it seems likely that PN response in this group may represent a ‘leaky’ mutation whereby provision of excess substrate allows synthesis of the product, PLP. In all three patients (1, 2 and 3) highly elevated concentrations of PLP, PL and PA are also present, similar to that seen in control individuals on vitamin B₆ supplementation.

B₆ vitamer profiles in normal individuals taking PLP supplementation have not been reported previously although from what is known of B₆ metabolic pathways, we would expect similar elevations of PLP, PL and PA to those on PN supplementation. Patients 11(b), 12, 13, 14(c) and 15 on PLP treatment who do not have PNPO deficiency have such a profile (Table 19 & 20). Thus the presence of PM, PMP, PN and PNP in high concentrations in the plasma of PNPO patients appears to allow differentiation from normal individuals on supplementation and certainly from a reference population not taking B₆.

Considering those patients in whom a known inborn error of B₆ metabolism has been excluded, but who show a response to PLP treatment (Patients 11(b), 12, 13, 14(c) and 15) different plasma profiles are evident (Tables 19 & 20). In particular the PLP:PL ratio is variable. This primarily reflects differing plasma PL concentrations, as PLP concentration is reasonably constant between patients. In patients 11(b) and 14(c) PLP:PL is significantly reduced and in patient 15 it is elevated compared to the reference range. Patients 13 and 14 have a PLP:PL ratio which lies within or close to the reference range.

As with all vitamers, plasma PL concentration is dependent on numerous factors. In patients on B₆ supplementation the dosing frequency, timing of sample in relation to B₆ dose (discussed in section 4.4.8 below) and length of treatment course may affect concentration. In the patients discussed here all had been on treatment for more than one month at the time of sampling, thus a steady state should have been reached. Plasma PL levels are also affected by the activity of tissue nonspecific alkaline phosphatase (TNSALP) and pyridoxal kinase (PK). TNSALP is known to be within
the normal range for all patients however PK has not been measured and deficiency of PK has yet to be described in patient groups.

4.4.3 Antiquitin deficiency

It is interesting to note that two siblings with pyridoxine dependent epilepsy due to antiquitin deficiency (Patients 4 & 5, Table 18) on PN therapy have reduced levels of PLP in plasma. Despite low PLP levels, PL and PA are significantly elevated leading to a considerably reduced PLP:PL ratio (Table 18). These two individuals also show elevation of PM, PMP and PN compared to the control population. In contrast patients 6 & 7 have a different profile with elevation in PLP, PL and PA and no other vitamers present. Clinically these children also differ in that Patients 4 & 5 suffered peripheral neuropathy secondary to PN therapy whereas Patients 6 & 7 have had no such complications.

One possible explanation for this apparent discrepancy in results may lie in differing biochemical phenotypes between families. PLP forms an adduct with piperideine-6-carboxylate, the equilibrium partner of α-AASA, which accumulates in antiquitin deficiency (Figure 5). The metabolic fate of this complex in vivo is not known however trichloroacetic acid used in the sample preparation (see Section 4.4.2) would be expected to liberate PLP from its complex with P6C. Patients 6 & 7 have more strongly elevated levels of α-AASA [28, 15 μmol/mmol creatinine] than patients 4& 5 [7.8, 7.4 μmol/mmol creatinine] which may be reflected in their differing plasma PLP concentrations. In vivo therefore, less plasma PLP may be available for catabolism to PA via PL in patients 6 & 7 as it is complexed with α-AASA, thus PL and PA are less strongly elevated. Significant elevation of PN, PMP and PM in patients 3 & 4 is suggestive of reduced PNPO activity, the cause of which is unclear, but it may represent an unrelated single nucleotide polymorphism within the PNPO gene resulting in mildly reduced enzyme activity that is overwhelmed by the amount of PNP being generated from the PN supplement.
4.4.4 Molybdenum Cofactor (MoCoF) and reduced activity of Aldehyde Oxidase (AOX)

Little is known about catabolic pathways of PLP in man and two patients (Patients 8 and 9, Table 18) with genetically confirmed MoCoF deficiency provide evidence for the role of the MoCoF dependent aldehyde oxidase in the formation of PA from PL. The absolute value of PA in plasma is reduced compared to the reference population, although the increased PL:PA ratio is most striking and likely to be a more reliable indicator as it is unlikely to be affected by B₆ intake from diet or medication (Table 18).

Patient 18 (Table 21) has a severe seizure disorder with neonatal onset which has clinical and biochemical features of a mitochondrial respiratory chain disorder (e.g. reduced complex IV activity in skin fibroblasts). Although it should be borne in mind that an age appropriate reference range is not available for this infant, the plasma vitamer profile showing increased plasma PL and an elevated PL: PA ratio is suggestive of aldehyde oxidase deficiency. MoCoF deficiency was however excluded as a cause of the seizure disorder in this patient thus other causes of reduced AOX activity should be considered. In the case of this patient, liver dysfunction secondary to mitochondrial disease is a possible cause.

4.4.5 Plasma B₆ vitamers in pregnancy

Human studies investigating the role of PLP in fetal development are understandably lacking. Based on our knowledge of the important functions of this vitamin in the central nervous system and the clinical effects evident in patients with inborn errors of metabolism resulting in B₆ depletion (e.g. PNPO deficiency), we can however assume that adequate provision of PLP to the developing foetus is essential.

The pregnant mother supplies B₆ to the fetus as pyridoxal which readily crosses the placenta in both directions, probably by a passive mechanism which is not saturable. Not surprisingly the transfer is much greater in the direction of the foetus (338). In comparison there is much less transport of PLP from the placenta to the fetus.

Plasma PLP concentrations are known to decrease in pregnant women early in gestation, from before the 16th week and return to pre-conceptual levels in the post-
partum period (339). Trumbo and Wang (340) showed that plasma concentrations of both PLP and PL fall during pregnancy and that their ratio is altered, with PLP decreasing to a larger degree than PL. This agrees with results from the pregnant subject (Patient 16; Table 21) reported here. In animal models, alkaline phosphatase levels are found to be reduced, thus it is surmised that placental PLP phosphatase activity may be responsible for the change (341).

4.4.6 Plasma B₆ vitamers and drug interactions

As discussed in section 1.1.14, PLP readily interacts with many compounds non-enzymatically. One well described example is the formation of an adduct between PLP and L-Dopa which results in inactivation of both compounds (147). Considering this, the B₆ profile of Patient 17 (Table 21) on Carbidopa (L-Dopa and peripheral decarboxylase inhibitor) is interesting as it shows a reduced concentration of PLP and PA and a low-normal concentration of PL, the PLP:PL ratio is slightly reduced illustrating that PLP is reduced to a greater extent than PL. This patient may benefit from pyridoxine therapy with careful clinical and biochemical monitoring.

4.4.7 Other applications of the LC-MS/MS method to patient populations

In addition to the disorders of B₆ metabolism discussed, this analytical method could be applied theoretically to other inborn errors on, or related to, this metabolic pathway. In hypophosphatasia absence of tissue non-specific alkaline phosphatase (TNSALP), results in an elevation of the phosphorylated B₆ vitamers in plasma (most importantly PLP) which are unable to cross cell membranes including those at the blood brain barrier. A corresponding reduction in pyridoxal which can cross cell membranes has been reported. Although no patient samples were available for inclusion in this work, theoretically a characteristic plasma B₆ profile would also be expected in this condition.

Pyridoxal kinase is required for the phosphorylation of un-phosphorylated vitamers, most importantly PL to PLP. Pyridoxal kinase activity is known to be markedly reduced in erythrocytes of African Americans compared to those of European ancestry but this is not associated with a clinical phenotype (64). A genetic deficiency of pyridoxal kinase causing human disease has not been described.
although the phenotype of brain pyridoxal kinase deficiency secondary to clock gene knockout has been demonstrated in animal models which suffer a lethal seizure disorder (41). A disturbed ratio of phosphorylated to non-phosphorylated plasma vitamers may aid identification of this theoretical disorder in targeted patient groups.

The ability to measure B\textsubscript{6} vitamers in plasma also provides important information about how normal concentrations and ratios are disturbed in individuals taking B\textsubscript{6} supplementation. The treatment doses used in the patients described here are large but are representative of normal clinical paediatric practice. A common feature to all cases is the massively elevated PL, PA and to a lesser extent PLP. This reflects the expected increased flux through intact catabolic pathways, however the effect of such supra-physiological concentrations in the long and short term is not known and warrants further study.

4.4.8 Timing of dose and plasma B\textsubscript{6} concentrations

In subjects on supplementation, plasma concentrations of PLP and PA are dependent on timing of the last B\textsubscript{6} dose. In healthy subjects given doses of up to 25 mg of PN, typically there is a rise in PL which peaks at 1-2 hours and then falls rapidly to baseline by 8 hours. In contrast PLP increases significantly by 1 hour and is well maintained for over 24 hours. Urinary PA appears more slowly and is cleared soon after, within 24 hours (342;343). Ubbink \textit{et al.} (344) showed that a PN dose of 100 mg leads to increased plasma PL and PLP concentrations which do not return to baseline within 48 hours, although the rate of reduction in PL is much greater than that seen for PLP.

Unfortunately accurate information about the timing of doses is not known for the patients reported here, however, it seems unlikely that timing of the dose will significantly affect either plasma PLP or PA given the maximum 12 hourly interval dosing regimen. In patients on low dose PN, plasma PL normalises quickly (within hours) but limited evidence suggests that this is not the case for larger doses (344), which is more relevant for the patients reported here.
4.4.9 Mega doses of vitamin B₆ and toxicity

Ingestion of large pyridoxine doses in humans is known to cause a severe sensory neuropathy which is dependent on dose and duration and usually reversible on stopping (93). Animal studies suggest that pyridoxine neuropathy is characterised by necrosis of the dorsal root ganglion sensory neurons and degeneration of both peripheral and central sensory projections (345). Important work in rats also shows that both protein deficiency and dehydration/oliguria may enhance the toxicity of pyridoxine, independent of the route of administration (162). Effects on the central nervous system can also be seen as, paradoxically, both pyridoxine (168) and pyridoxal phosphate (131) may be pro-convulsant when used in neonates and young infants in clinical practice.

The mechanisms of vitamin B₆ toxicity have not been fully elucidated. Levine et al. (162) showed that PL and PLP are more lethal to rats than PN or PM, although the typical clinical and histological evidence of toxicity (severe damage to primary sensory neurones that mimic axonal reaction with central chromatolysis) usually associated with PN were not seen. In contrast equal toxicity of PN, PM and PL was seen in dorsal root ganglia cell culture (346). The question of which, if any, of the vitamers are more toxic and the resulting implications for patient treatment and monitoring remains unanswered. As all vitameric forms are ultimately metabolised to the cofactor PLP before catabolism to PA via PL, and most patients metabolise exogenous PN and PLP without any significant increase in plasma PN level, in man it seems unlikely that PN per se is responsible for toxicity. It is, however, possible theoretically that toxicity of mega B₆ doses affects only a subgroup of the population in whom there is a relatively reduced PNPO activity where elevated plasma PN is observed on treatment. Further research is needed and measurement of all B₆ vitamers in plasma and other body fluids should form an important part of this. This work also indicates that clinicians should consider monitoring nerve conduction in patients on both PN and PLP therapy.
4.5 SUMMARY

In summary, LC-MS/MS measurement of B₆ vitamers in plasma is a valid, quick and practical method for use in various patient groups. It is a valuable tool in the battery of investigations for childhood and neonatal epilepsy and may aid diagnosis in B₆ dependent seizure disorders, including in patients already commenced on treatment.

Further B₆ vitamer data should be gathered in patient groups on B₆ supplementation to advance understanding of possible toxic mechanisms of this therapy which is routinely used in large numbers of adult and paediatric populations.
Chapter 5
Vitamin B₆ and serotonin metabolism in autism
5.1 Introduction

Autism is a common neurodevelopmental disorder characterised by difficulties in social communication, reciprocal social interaction and imaginary thought accompanied by restrictive, stereotyped behaviours. Up to one third of patients have associated co-morbidities including seizures, cognitive impairment and sleep disorders (239). Individuals with Asperger’s syndrome share many of these features but have preserved language and cognitive function.

At present the diagnosis is solely clinical and is made by a multi-disciplinary team using detailed neuropsychological assessments which should include the input of family, carers and teachers. Standardised criteria such as the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) are widely used and provide consensus for diagnosis around the world. Of note, the diagnostic features must have onset before 3 years of age recognising the developmental nature of this disorder.

Numerous epidemiological studies provide compelling evidence for a genetic basis (347;348), but despite intense research activity the aetiology of autism remains elusive as do effective treatments. It is appealing to consider that regardless of genetic basis, a vast complexity of differing underlying molecular mechanisms may converge on a final common biochemical pathway which is likely to be of particular importance during early neurodevelopment.

Thus an alternative approach to understanding the pathophysiology of autistic spectrum disorders is from a biochemical perspective. More than 50 years ago elevated whole blood serotonin or hyperserotonaemia was first described in infantile autism (228). Subsequently more than 25 studies have reproduced this observation, where typically the concentration of whole blood or platelet serotonin is found to be 50% higher than normal, in one third of subjects (349).

However, the underlying mechanism of hyperserotonaemia and how it may relate to the clinical phenotype remains unknown (348). Very few studies have investigated whole blood serotonin in patients with high functioning autism and Asperger’s syndrome and they represent an interesting group for further work.
In the central nervous system serotonin is a neurotransmitter whose role is implicated in many and varied aspects of behaviour including mood, sleep, and appetite. In addition, serotonergic neurones are thought to play a vital role in the developing nervous system, often playing a neuro-modulatory function. If serotonin is indeed implicated in the development of autism, its concentration in the central nervous system (CNS) is more likely to be of relevance than in the periphery, although the incomplete blood brain barrier during fetal development and resulting exposure of the developing brain to peripherally produced serotonin should be borne in mind.

Work examining central serotonin metabolism in autistic subjects however is inconclusive with the metabolite of serotonin (5-HIAA) measured in CSF often showing no difference to control subjects (350). This may reflect small patient numbers, poorly defined phenotypes and the fact that measurement of CSF 5-HIAA concentration is a crude approach to examine what may be small regional variations in serotonin metabolism.

Serotonin is synthesised from the essential amino acid tryptophan which competes with the other large neutral amino acids (Leucine, Leu; Isoleucine, Ile; Valine, Val; Tyrosine, Tyr; Phenylalanine, Phe) to cross the blood brain barrier via the L-type amino acid transporter (LAT1). The two step metabolic pathway is shown in Figure 30 where the rate limiting step in synthesis is tryptophan hydroxylase (TPH). Under normal physiological conditions this enzyme is not fully saturated, thus an increase in tryptophan concentration may result in increased 5-hydroxytryptophan (5-HTP) and serotonin production (188). Aromatic amino acid decarboxylase (AADC) catalyses the second and final step which synthesises serotonin from 5-HTP and requires pyridoxal phosphate (PLP) as a cofactor. This stage can become rate-limiting in B6 deficiency states or when AADC activity is very low, for example secondary to a genetic deficiency (351).

Autistic spectrum disorders, serotonin and vitamin B6 metabolism have been discussed in depth in Sections 1.1, 1.2 and 1.3.
**Figure 30. Synthetic pathways of serotonin and dopamine**

(i) tryptophan hydroxylase (ii) aromatic amino acid decarboxylase (iii) tyrosine hydroxylase

5.2 **HYPOTHESIS**

A proportion of individuals with high-functioning autism and Asperger’s syndrome will, like a significant proportion of children at the more severe end of the spectrum, show hyperserotoninaemia. Investigation of plasma levels of amino acids (particularly tryptophan), AADC activity and PLP status may provide clues to the biochemical derangement.

5.3 **AIM**

To explore the relationship of whole blood serotonin, plasma B$_6$ vitamers (including pyridoxal phosphate), tryptophan and AADC activity in a well-defined autistic cohort and compare results to age, gender and ethnically matched controls. Any abnormality detected at a biochemical level will then direct targeted gene sequencing.
5.4 **STUDY DESIGN**

The study is a case-control design. Whole blood serotonin may be affected by age, gender and ethnicity (229;349) thus control subjects were selected to match for these variables as far as possible. Some studies suggest that serotonin may be influenced by time of day (352) therefore all samples were collected within a six hour period, in the late morning to afternoon.

Pyridoxal phosphate concentrations are known to be affected during periods of acute infection and inflammation (89) so the participants recruited were not systemically unwell at the time of sampling or in preceding days. As vitamin B₆ concentrations in plasma reflect dietary intake, both autistic and control patients were asked to complete a 3-Day Food Diary to allow estimation of B₆ intake and to post it back to the researcher in a stamped addressed envelope.

Whole blood serotonin concentration is influenced by the concentration of its precursor tryptophan which in turn is related to other plasma amino acids. Additionally plasma tryptophan is extensively bound to albumin, thus the ‘free’, biologically active form is affected by serum albumin concentrations. A full plasma amino acid profile and albumin concentration was measured alongside whole blood serotonin in each autistic subject and in control subjects when sample was sufficient.

5.5 **METHODS**

5.5.1 **Subject recruitment**

5.5.1.1 **Autistic subjects**

Patients with autistic spectrum disorder were recruited from the Social and Communication Disorders Clinic at Great Ormond Street Hospital for Children, London. This clinic accepts referrals from Consultant Paediatricians and Child Psychiatrists for assessment of children and young adults in whom a diagnosis of ‘high functioning’ autism is suspected and a second opinion is required. Where patients met inclusion criteria (Table 22) consent was taken from young adults and
their parents and assent taken from children too young to give full consent. Samples were collected following psychological assessment.

A small number of families approached the research team as a result of publicity on the Great Ormond Street Hospital website; those who met inclusion criteria (Table 22) were recruited into the study and samples were collected.

All children were assessed using one or more standardised, validated diagnostic tools described in Section 5.5.1.2. Samples from those in whom a diagnosis of autistic spectrum disorder was not reached according to diagnostic criteria were not included in the subsequent analysis. Other relevant medical and neurodevelopmental information was collected from the clinical notes and parental interview.

Table 22. Inclusion and exclusion criteria for autistic and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autism</td>
<td>Cases of all ages and both sexes from any ethnic background who fulfil DSM IV and ICD-10 criteria for the diagnosis of Autistic Spectrum Disorder based on standardised assessments, for example Autism Diagnostic Observation Schedule (ADOS) and The Developmental, Dimensional and Diagnostic Interview (3Di)</td>
<td>Any child or family where an accurate diet and medication history is not available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Any case with intercurrent illness, defined as systemically unwell or febrile within the previous 48 hours</td>
</tr>
<tr>
<td>Controls</td>
<td>Age and gender matched to cases</td>
<td>Any case with intercurrent illness, defined as systemically unwell or febrile within the previous 48 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Any case who has a medical diagnosis or chronic illness (diagnosed or under investigation) which may confound interpretation of results</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Any child or family where an accurate diet and medication history is not available</td>
</tr>
</tbody>
</table>
5.5.1.2 Measures

The Developmental, Dimensional and Diagnostic Interview (3Di)

The 3Di is a validated, parent-report computerized interview which emulates the Autism Diagnostic Interview – Revised (ADI-R) diagnostic algorithms and also measures the associated features of autism (240). Like the ADI-R, the 3Di uses ICD-10 and DSM-IV-TR diagnostic guidelines for autistic spectrum disorders. The 3Di takes a developmental history and a selection of 122 questions concerning both current and past symptoms both of which contribute to diagnostic algorithms which give scores in the three domains of social interaction, communication, and repetitive and stereotyped behaviour (RSB).

In addition to its ADI-R equivalent pervasive developmental disorders (PDD) algorithm, the 3Di also includes scales for assessing the following associated features of autism: Fine Motor Impairment, Visuo-Spatial Impairment, Gross Motor Impairment; Auditory Sensitivity; Feeding Difficulties and Sleep Problems.

The Autism Diagnostic Observation Schedule (ADOS)

The ADOS (353) is a semi-structured observational assessment, which measures social interaction, communication, repetitive behaviour and imagination for individuals with suspected PDD. It comprises four modules tailored to an individual’s language ability. The algorithm uses selective social communication and reciprocal social interaction scores to generate a total score for each domain. Elevated scores place an individual in the autism spectrum or autism diagnostic range, depending on the severity and frequency of the behaviours displayed. All ADOS were scored from video recordings.

The Strengths and Difficulties Questionnaire (SDQ)

The SDQ comprises 25 items in 5 subscales: conduct problems, emotional problems, hyperactivity, peer problems and prosocial behaviour (354). Rated by parents and by teachers, it has been shown to possess acceptable reliability and validity when assessing adaptation and psychopathology in children and adolescents both as a screening instrument (355) and as a dimensional measure (356).
**Intelligence Quotient (IQ)**

IQ data were collected as part of clinical assessment over the time frame of the study, and as such a variety of measures were used. Instruments included the British Picture Vocabulary Scale, the Wechsler Abbreviated Scale of Intelligence and the Wechsler Intelligence Scale for Children – Third and Fourth Editions. Summary variables were computed from these scores, for verbal and performance IQ, standardized to have a mean of 100 and a standard deviation of 15. In the current study verbal IQ data were available for all participants, and performance IQ data were available for 84% of the sample. This reflects changes in clinic practice over time rather than any tendency to conduct performance IQ testing depending upon a particular child’s presenting difficulties.

Final consensual clinical diagnosis was based upon information from the 3Di, plus (where available) the Autism Diagnostic Observation Schedule (353), and structured reports from the child’s nursery or school. Due to the difficulties of using DSM criteria to distinguish Asperger’s syndrome from autistic disorder, Szatmari (2000) (357) guidelines were used to differentiate these according to whether or not there was a delay in the onset of spoken language. Thus, for a diagnosis of autism, scores above the standard 3Di cut-points in reciprocal social interaction, communication and repetitive, stereotyped behaviour (RSB) were required, as well as delayed development of onset of single word (>24 months) or phrase speech (>36 months). Asperger’s syndrome was diagnosed in the presence of above-threshold 3Di scores for reciprocal social interaction, communication and RSBs, without a delay in either single-word or phrase speech.

**5.5.1.3 Control subjects**

Age, gender and ethnically matched controls were recruited from outpatient clinics at Great Ormond Street Hospital for Children. Clinical notes were reviewed and parents interviewed to ensure that these subjects had no diagnosis or family history of a neurodevelopmental disorder (including autism) and consumed a healthy diet at the time of sampling. Where applicable, medication history was documented. Inclusion and exclusion criteria for autistic subjects and controls are shown in Table 22.
5.5.1.4 Sample size and power calculation

For 90% power to detect a difference in whole blood serotonin, 22 matched pairs were required.

5.5.1.5 Ethical approval

Ethical approval for this study was granted by the Riverside Research Ethics Committee (09/H0706/85).

5.5.2 Collection and handling of samples

Subjects and controls provided a single blood sample for analysis of biochemical parameters as detailed in Table 23. The time of day and relation to food intake was documented.

Table 23. Collection and handling of blood samples for analytes measured in autistic and control subjects

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sample and handling</th>
<th>Sample bottle</th>
<th>Sample volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal 5’-phosphate (HPLC analysis)</td>
<td>Plasma protected from light, stored frozen -80°C</td>
<td>EDTA</td>
<td>1-2</td>
</tr>
<tr>
<td>B6 vitamers and 4-pyridoxic acid (LC-MS/MS analysis)</td>
<td>Plasma protected from light, stored frozen -80°C</td>
<td>EDTA</td>
<td>1-2</td>
</tr>
<tr>
<td>Whole blood serotonin</td>
<td>Whole blood, frozen at -70°C at the bedside</td>
<td>EDTA with ascorbic acid</td>
<td>2</td>
</tr>
<tr>
<td>Plasma amino acids and albumin</td>
<td>Plasma frozen at -70°C or analysed immediately</td>
<td>Lithium Heparin</td>
<td>1</td>
</tr>
<tr>
<td>Aromatic aminoacid decarboxylase (dopa decarboxylase) activity</td>
<td>Plasma frozen at -80°C</td>
<td>Lithium Heparin or EDTA</td>
<td>1</td>
</tr>
<tr>
<td>*DNA</td>
<td>Whole blood. DNA extracted and frozen at -20°C</td>
<td>EDTA</td>
<td>2</td>
</tr>
</tbody>
</table>

* Sample collected in autistic subjects only
5.5.3 Laboratory methods

Whole blood serotonin was analysed by HPLC with fluorescence detection in the Neurometabolic Diagnostic Laboratory, National Hospital for Neurology and Neurosurgery, Queen Square, London (289) (Section 2.2.4). The whole blood serotonin samples were analysed in six batches over a period of 24 months; each sample was analysed within 4 months of collection, prior to which it was stored at -80°C.

All B₆ vitamers were measured in plasma using HPLC linked to tandem mass spectrometry as described in Section 2.2.2. Each sample was spun within 1 hour of collection and plasma frozen at -80°C. The samples were then analysed in 6 batches over a period of 6 months and prior to analysis were stored at -80°C for between 1 and 16 months.

Plasma PLP was also analysed by a HPLC method using a Chromsystems® kit in the Neurometabolic Diagnostic Laboratory, National Hospital for Neurology and Neurosurgery, Queen Square, London, as described in Section 2.2.1.

Plasma aromatic aminoacid decarboxylase (AADC) activity was measured at the Institute of Neurology, Queen Square, London using an assay that measures the conversion of L-dopa into dopamine by AADC present in human plasma (288) (Section 2.2.3). Since AADC activity is much higher with L-dopa as a substrate, this method is preferred over activity measurement using 5-HTP as a substrate for diagnostic purposes (358).

Briefly, plasma is first incubated for 120 minutes with the cofactor pyridoxal 5’-phosphate. In the second stage L-dopa is added to the reaction mixture and incubated for 90 minutes to allow AADC to convert L-dopa into dopamine. The reaction is stopped with perchloric acid to precipitate the protein in the sample and the protein is removed by centrifugation. The sample is then injected onto a C18 reverse-phase HPLC column and dopamine present in the sample is measured by electrochemical detection.

Samples were analysed in four batches over a period of 22 months. Samples were collected and stored at -80 °C for a maximum of 8 months before analysis.
Plasma amino acids and albumin were measured on the day of sampling in the chemical pathology laboratories, Great Ormond Street Hospital, London by HPLC or frozen at -70°C pending this analysis.

5.5.4 Statistical analysis

Data was tested for normal distribution using graphical representation and the Kolmogorov-Smirnov test.

Normally distributed data was subsequently analysed using parametric tests (Student’s t-test) and data that were not normally distributed were analysed using non-parametric (Mann-Whitney U) tests to evaluate differences between patient and control groups. Linear regression analysis and calculation of correlation coefficients (parametric data, Pearson r and non-parametric data, Spearman r) were used to investigate the relationship between variables.

Some analytes were also compared to a laboratory reference range in addition to age matched controls. In particular the upper reference limit for whole blood serotonin in the National Hospital for Neurology and Neurosurgery Laboratory was used in a Fisher’s exact test to compare proportions. This reference range was historically devised using disease controls, (paediatric and adult patients without neurological disease) and healthy adult volunteers.

5.5.6 Analysis of Food Diary

This was not undertaken as insufficient numbers were returned.

5.6 RESULTS

5.6.1 Demographic and clinical characteristics of case and control populations

Table 24 shows demographic information, clinical diagnosis and regular medications for case and control groups.

20 cases were recruited from the Social and Communication Disorders clinic and 3 from advertisement. Of the 20 children recruited via the Social and Communication
Disorders clinic, one was subsequently excluded as he did not fulfil diagnostic criteria for autistic spectrum disorder or Asperger’s syndrome.

The control and autistic groups were matched for age and an unpaired t-test showed there was no statistically significant difference between them (p = 0.53) (Figure 31 and Table 24). 21 of the 22 cases with autistic spectrum disorder were matched for gender; 19 of the 22 cases were matched for ethnicity.

All blood and urine samples in the autistic and control subjects were collected between 11:00 and 17:00.

The food diary was returned in only a small number of the control group (5 out of 22 control subjects and 13 out of 22 autism subjects) therefore it was not meaningful to analyse dietary intake in the autistic or control group as a whole.
Table 24. Demographic, diagnostic and medication information for autistic and control groups

<table>
<thead>
<tr>
<th></th>
<th>Autism</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>19M, 3F</td>
<td>18M, 4F</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>20 Caucasian; 2 Asian</td>
<td>19 Caucasian, 2 Asian, 1 Turkish</td>
</tr>
<tr>
<td><strong>Age range</strong> (months)</td>
<td>54 – 222</td>
<td>52 - 216</td>
</tr>
<tr>
<td><strong>Mean (months)</strong></td>
<td>144.1</td>
<td>152.5</td>
</tr>
<tr>
<td><strong>Median (months)</strong></td>
<td>143.0</td>
<td>162.5</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>41.5</td>
<td>45.1</td>
</tr>
<tr>
<td><strong>Standard error</strong></td>
<td>8.8</td>
<td>9.6</td>
</tr>
<tr>
<td><strong>Regular medications and nutritional supplements</strong></td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Multivitamin 1</td>
<td>HMG CoA reductase inhibitor 4</td>
</tr>
<tr>
<td></td>
<td>Cod liver oil 2</td>
<td>Amlodipine 1</td>
</tr>
<tr>
<td></td>
<td>Vitamin C 1</td>
<td>Trimethoprim 1</td>
</tr>
<tr>
<td></td>
<td>Clonidine 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sodium valproate 1</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical diagnosis</strong></td>
<td>See Table 22</td>
<td>Familial hypercholesterolaemia 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed hyperlipidaemia 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urological or structural renal abnormalities 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium chain acyl-CoA dehydrogenase deficiency 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α1-Antitrypsin deficiency 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactosaemia 1</td>
</tr>
</tbody>
</table>

M – male; F - female
Figure 31. Graph showing that there is no statistically significant difference in age distribution between the autistic and control group

$p = 0.53, \text{ns}$
5.6.2 Whole blood serotonin in autistic and control populations

Results for autistic subjects and controls are shown in Table 25 and Figures 32 and 33. Whole blood serotonin concentration is normally distributed in both groups and passed the Kolmogorov-Smirnov test for normality.

Table 25. Whole blood serotonin concentration in the autistic and control group showing the range, mean, median, standard deviation and standard error

<table>
<thead>
<tr>
<th></th>
<th>Autism</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Range (nmol/L)</td>
<td>539 - 3265</td>
<td>485 - 2792</td>
</tr>
<tr>
<td>Mean (nmol/L)</td>
<td>1646</td>
<td>1406</td>
</tr>
<tr>
<td>Median (nmol/L)</td>
<td>1560</td>
<td>1411</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>618.1</td>
<td>561.5</td>
</tr>
<tr>
<td>Standard error</td>
<td>131.8</td>
<td>119.7</td>
</tr>
</tbody>
</table>
Figure 32. Normal distribution of whole blood serotonin concentration in autistic group (A) and control group (B)
The two groups were compared statistically using an unpaired t-test; there was no significant difference in whole blood serotonin concentration between the autistic and control group as shown in Figure 33 (p = 0.18).

![Graph showing comparison of whole blood serotonin concentration in autistic group and control group](image)

**Figure 33. Comparison of whole blood serotonin concentration in autistic group and control group**

The proportion of subjects with elevated serotonin within each population was also compared using a two-tailed Fisher’s exact test. Whole blood serotonin results were converted into categorical data by using the upper limit of the laboratory normal reference range as a cut-off; a value for whole blood serotonin of >1600 nmol/L was considered to be elevated and ≤1600 nmol/L as not elevated. Results of two-tailed Fisher’s exact test (Table 26) showed no difference between the autistic and control population (p=0.3475). This data is presented graphically in Figure 34.
Table 26. Data for two-tailed Fisher’s exact test of whole blood serotonin concentration in autistic and control groups

<table>
<thead>
<tr>
<th>Whole blood serotonin (nmol/L)</th>
<th>Autism</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1600 (elevated)</td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>&lt;1600 (not elevated)</td>
<td>12</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>22</td>
<td>44</td>
</tr>
</tbody>
</table>

Figure 34. Distribution of whole blood serotonin concentration by age in both autistic and control subjects relative to the laboratory upper reference limit (1600 nmol/L)
The relationship between age and whole blood serotonin was examined in the autistic and control group by linear regression analysis and is represented graphically in Figures 35A and 35B, respectively. There is a trend to decreasing whole blood serotonin concentration with age in the autistic population, although this does not reach statistical significance (Pearson $r = -0.38$, $r^2 = 0.38$, $p=0.08$). There is no apparent relationship between whole blood serotonin concentration and age in the control group (Pearson $r = 0.002$, $r^2 = 0.00$, $p=0.99$).
Figure 35. Distribution of whole blood serotonin by age in autistic group (A) and control group (B)

A trend to reducing concentration of whole blood serotonin with age is seen in the autistic group (Pearson r = -0.38, r² = 0.38, p=0.08); no relationship is evident in the control group (Pearson r = 0.002, r² = 0.00, p=0.99).
5.6.3 Plasma albumin and amino acids in autistic and control populations

Plasma albumin and amino acids were measured in 21 of 22 autistic subjects; the sample was insufficient in one case. Due to limited sample availability, sufficient sample to measure albumin and amino acids was available in only 9 of 22 control subjects. Results are shown in Tables 27-29 and compared to the laboratory paediatric reference range. Due to the small numbers in the control group, non-parametric statistical tests were applied.

It was found that albumin concentration was normal in all subjects (data not shown).

A significant difference between the autistic and control population was evident for plasma glycine and taurine; there was no statistically significant difference for the other amino acids.
Table 27. Plasma amino acid concentrations in autistic and control populations (A)

<table>
<thead>
<tr>
<th>Paediatric reference range</th>
<th>Glycine</th>
<th>Serine</th>
<th>Threonine</th>
<th>Proline</th>
<th>Leucine</th>
<th>Isoleucine</th>
<th>Valine</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100-330</td>
<td>90-290</td>
<td>70-220</td>
<td>85-290</td>
<td>65-220</td>
<td>26-100</td>
<td>90-300</td>
<td>150-450</td>
</tr>
<tr>
<td>Autism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>509</td>
<td>379</td>
<td>225</td>
<td>135</td>
<td>317</td>
<td>198</td>
<td>115</td>
<td>356</td>
</tr>
<tr>
<td>Mean (nmol/L)</td>
<td>253</td>
<td>209.7</td>
<td>130.5</td>
<td>110.7</td>
<td>207.6</td>
<td>126.6</td>
<td>69.6</td>
<td>231.6</td>
</tr>
<tr>
<td>Median (nmol/L)</td>
<td>227</td>
<td>194</td>
<td>122</td>
<td>116</td>
<td>189</td>
<td>118</td>
<td>66</td>
<td>216</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>82.9</td>
<td>67.9</td>
<td>35.2</td>
<td>18.4</td>
<td>49.4</td>
<td>54.6</td>
<td>19.9</td>
<td>56.7</td>
</tr>
<tr>
<td>Standard error</td>
<td>18.1</td>
<td>22.6</td>
<td>7.7</td>
<td>6.1</td>
<td>10.8</td>
<td>8.3</td>
<td>6.9</td>
<td>12.4</td>
</tr>
<tr>
<td>p value</td>
<td>0.04*</td>
<td>0.07</td>
<td>0.26</td>
<td>0.22</td>
<td>0.87</td>
<td>0.68</td>
<td>0.47</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*indicates a significant difference, p<0.05
Table 28. Plasma amino acid concentrations in autistic and control populations (B)

<table>
<thead>
<tr>
<th>Paediatric reference range</th>
<th>Glutamine</th>
<th>Arginine</th>
<th>Ornithine</th>
<th>Lysine</th>
<th>Methionine</th>
<th>Taurine</th>
<th>Phenylalanine</th>
<th>Tyrosine</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (nmol/L)</td>
<td>Mean</td>
<td>Median</td>
<td>Standard deviation</td>
<td>Standard error</td>
<td>p value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>408 – 654</td>
<td>547.5</td>
<td>551</td>
<td>66.5</td>
<td>14.5</td>
<td>0.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>466 – 627</td>
<td>552</td>
<td>553</td>
<td>60.3</td>
<td>20.1</td>
<td>0.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 – 136</td>
<td>73.4</td>
<td>69</td>
<td>23.6</td>
<td>5.2</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58 – 99</td>
<td>73.2</td>
<td>71</td>
<td>12.8</td>
<td>4.3</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 – 166</td>
<td>86.4</td>
<td>85</td>
<td>27.9</td>
<td>6.1</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 – 105</td>
<td>75.1</td>
<td>76</td>
<td>19.5</td>
<td>6.5</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95 – 273</td>
<td>170</td>
<td>163</td>
<td>40.9</td>
<td>8.9</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102 – 250</td>
<td>175.6</td>
<td>174</td>
<td>46.5</td>
<td>15.5</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 – 44</td>
<td>25.7</td>
<td>22</td>
<td>8.3</td>
<td>1.8</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 – 34</td>
<td>24.1</td>
<td>23</td>
<td>5.4</td>
<td>1.8</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>39 – 132</td>
<td>65.1</td>
<td>59</td>
<td>20.8</td>
<td>4.5</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 – 64</td>
<td>50.2</td>
<td>59</td>
<td>8.9</td>
<td>2.9</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 – 91</td>
<td>61.9</td>
<td>55</td>
<td>12.6</td>
<td>2.7</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 – 86</td>
<td>60.9</td>
<td>63</td>
<td>14.3</td>
<td>4.8</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 - 102</td>
<td>66.7</td>
<td>60</td>
<td>17</td>
<td>3.7</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49- 99</td>
<td>67.8</td>
<td>67</td>
<td>16.6</td>
<td>5.5</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*indicates a significant difference, p<0.05
Table 29. Plasma amino acid concentrations in autistic and control populations (C)

<table>
<thead>
<tr>
<th>Paediatric reference range</th>
<th>Tryptophan</th>
<th>Histidine</th>
<th>Asparagine</th>
<th>Aspartate</th>
<th>Glutamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30-80</td>
<td>30-150</td>
<td>15 - 83</td>
<td>1 - 17</td>
<td>25-130</td>
</tr>
<tr>
<td>Range (nmol/L)</td>
<td>Autism</td>
<td>Control</td>
<td>Autism</td>
<td>Control</td>
<td>Autism</td>
</tr>
<tr>
<td>Mean (nmol/L)</td>
<td>52</td>
<td>60.7</td>
<td>89.1</td>
<td>81</td>
<td>56.4</td>
</tr>
<tr>
<td>Median (nmol/L)</td>
<td>61</td>
<td>56</td>
<td>87</td>
<td>76</td>
<td>51</td>
</tr>
<tr>
<td>Standard deviation (nmol/L)</td>
<td>11.3</td>
<td>13.6</td>
<td>11.9</td>
<td>13.6</td>
<td>11.4</td>
</tr>
<tr>
<td>Standard error</td>
<td>2.5</td>
<td>4.5</td>
<td>2.6</td>
<td>4.5</td>
<td>2.6</td>
</tr>
<tr>
<td>p value</td>
<td>0.98</td>
<td>0.21</td>
<td>0.23</td>
<td>0.09</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Tryptophan competes with other large neutral amino acids (LNAA) to enter the central nervous system via the LNAA transporter (LAT1), thus the concentration of tryptophan relative to other LNAA (Leu, Ile, Val, Tyr, Phe) is of importance for central serotonin synthesis.

The tryptophan: large neutral amino acid ratio (Trp: LNAA) for the autistic and control groups are presented in Tables 30 and 31. There is no significant difference between autistic and control populations for total LNAA concentration (p = 0.94) or Trp: LNAA ratio (p = 0.80).

**Table 30. Range, mean, median, standard deviation and standard error of the plasma concentration of large neutral amino acids [Leu, Ile, Val, Tyr, Phe], tryptophan and the ratio of Trp: LNAA in autistic patients**

<table>
<thead>
<tr>
<th></th>
<th>Total LNAA (nmol/L)</th>
<th>Trp (nmol/L)</th>
<th>Trp:LNAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>376 – 790</td>
<td>27 – 73</td>
<td>0.07 – 0.14</td>
</tr>
<tr>
<td>Mean</td>
<td>556</td>
<td>52</td>
<td>0.12</td>
</tr>
<tr>
<td>Median</td>
<td>556</td>
<td>61</td>
<td>0.11</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>123.6</td>
<td>11.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Standard error</td>
<td>26.9</td>
<td>2.5</td>
<td>0.03</td>
</tr>
</tbody>
</table>

LNAA = large neutral amino acids; Trp = tryptophan

**Table 31. Range, mean, median, standard deviation and standard error of the plasma concentration of large neutral amino acids [Leu, Ile, Val, Tyr, Phe], tryptophan and the ratio of Trp: LNAA in control patients**

<table>
<thead>
<tr>
<th></th>
<th>Total LNAA (nmol/L)</th>
<th>Trp (nmol/L)</th>
<th>Trp:LNAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>455-861</td>
<td>49-88</td>
<td>0.08-0.13</td>
</tr>
<tr>
<td>Mean</td>
<td>571.3</td>
<td>60.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Median</td>
<td>544.0</td>
<td>56.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>131.9</td>
<td>13.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Standard error</td>
<td>43.9</td>
<td>4.5</td>
<td>0.004</td>
</tr>
</tbody>
</table>

LNAA = large neutral amino acids; Trp = tryptophan
5.6.3.1 The relationship of whole blood serotonin with large neutral amino acids

Figures 36 & 37 and Tables 32 & 33 show the results of correlation and linear regression analysis of whole blood serotonin and plasma large neutral amino acids in the autistic and control group.

In the autism group a positive correlation is evident for all amino acids except valine and the relationship is statistically significant for tryptophan, tyrosine and phenylalanine. In the control group no statistically significant correlation is evident.

**Table 32. Pearson correlation coefficient (r), r² and p value of linear regression of whole blood serotonin concentration and plasma large neutral amino acids in autistic population**

<table>
<thead>
<tr>
<th>Graph</th>
<th>Amino acid</th>
<th>Pearson r</th>
<th>r²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tryptophan</td>
<td>0.62</td>
<td>0.39</td>
<td>0.002</td>
</tr>
<tr>
<td>B</td>
<td>Tyrosine</td>
<td>0.51</td>
<td>0.264</td>
<td>0.017</td>
</tr>
<tr>
<td>C</td>
<td>Phenylalanine</td>
<td>0.55</td>
<td>0.30</td>
<td>0.01</td>
</tr>
<tr>
<td>D</td>
<td>Isoleucine</td>
<td>0.34</td>
<td>0.11</td>
<td>ns</td>
</tr>
<tr>
<td>E</td>
<td>Valine</td>
<td>0.06</td>
<td>0.003</td>
<td>ns</td>
</tr>
<tr>
<td>F</td>
<td>Leucine</td>
<td>0.40</td>
<td>0.16</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant, i.e. p >0.05

**Table 33. Spearman correlation coefficient (r), r² and p value of linear regression of whole blood serotonin concentration and plasma large neutral amino acids in control population**

<table>
<thead>
<tr>
<th>Graph</th>
<th>Amino acid</th>
<th>Spearman r</th>
<th>r²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tryptophan</td>
<td>-0.03</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>B</td>
<td>Tyrosine</td>
<td>0.05</td>
<td>0.03</td>
<td>ns</td>
</tr>
<tr>
<td>C</td>
<td>Phenylalanine</td>
<td>0.30</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td>D</td>
<td>Isoleucine</td>
<td>0.25</td>
<td>0.05</td>
<td>ns</td>
</tr>
<tr>
<td>E</td>
<td>Valine</td>
<td>0.10</td>
<td>0.06</td>
<td>ns</td>
</tr>
<tr>
<td>F</td>
<td>Leucine</td>
<td>0.46</td>
<td>0.14</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant, i.e. p >0.05
Figure 36. Relationship of whole blood serotonin to large neutral amino acids in plasma of autistic population

A = tryptophan; B= tyrosine; C = phenylalanine; D= isoleucine; E = valine; F = leucine
Figure 37. Relationship of whole blood serotonin to large neutral amino acids in plasma of the control population

A = tryptophan; B = tyrosine; C = phenylalanine; D = isoleucine; E = valine; F = leucine.
5.6.4 Plasma B<sub>6</sub> vitamers in the autistic and control population

Results for plasma B<sub>6</sub> vitamers in 21 autistic subjects and 21 control subjects are shown in Table 34 and Figure 38. There was insufficient sample available to analyse B<sub>6</sub> vitamers in control subject 10 and plasma pyridoxic acid (PA) was not quantifiable in control subject 13. One subject in the autistic group was taking an over the counter multivitamin preparation containing pyridoxine, this sample was not included in the analysis. Each plasma sample was run in duplicate and the mean concentration is presented.

Groups were compared statistically using the Mann Whitney U test for non-parametric data as some data were not normally distributed according to the Kolmogorov-Smirnov (KS) test. No statistical difference was observed between groups for total B<sub>6</sub> (sum of PLP, PMP, PNP, PL, PA, PN and PM), pyridoxal phosphate (PLP), pyridoxic acid (PA), pyridoxine (PN) or pyridoxamine phosphate (PMP). Plasma PL was significantly lower in the autistic group compared to controls. Pyridoxine phosphate (PNP) and pyridoxamine (PM) were not detectable in any subject.

![Figure 38. Graph showing total plasma vitamin B<sub>6</sub> concentration in autistic and control groups](image)

\[ p = 0.21 \]
Table 34. Plasma B₆ vitamer concentrations in autistic and control populations showing range, mean, median, standard deviation, standard error, results of Kolmogorov-Smirnov (KS) normality test and p value of Mann Whitney U

<table>
<thead>
<tr>
<th></th>
<th>PLP</th>
<th>PL</th>
<th>PA</th>
<th>PMP</th>
<th>PN</th>
<th>PNP</th>
<th>PM</th>
<th>Total B₆</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total number</strong></td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td><strong>Range (nmol/L)</strong></td>
<td>46.8 – 206.2</td>
<td>46.4 – 350.1</td>
<td>4.2 – 18.6</td>
<td>5.8 – 30.1</td>
<td>16.0 – 119.0</td>
<td>17.6 – 123.2</td>
<td>nd - 9.1</td>
<td>nd - 9.3</td>
</tr>
<tr>
<td><strong>Mean (nmol/L)</strong></td>
<td>100.2</td>
<td>139.9</td>
<td>8.8</td>
<td>12.7</td>
<td>38.3</td>
<td>53.0</td>
<td>3.5</td>
<td>2.54</td>
</tr>
<tr>
<td><strong>Median (nmol/L)</strong></td>
<td>90.4</td>
<td>106.4</td>
<td>7.1</td>
<td>12.0</td>
<td>29.2</td>
<td>46.8</td>
<td>3.5</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>42.0</td>
<td>91.1</td>
<td>3.7</td>
<td>6.3</td>
<td>23.5</td>
<td>28.6</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Standard error</strong></td>
<td>9.2</td>
<td>19.9</td>
<td>0.8</td>
<td>1.4</td>
<td>5.1</td>
<td>6.4</td>
<td>0.5</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Passed KS normality test?</strong></td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td>0.30</td>
<td>0.04*</td>
<td>0.07</td>
<td>0.09</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Y – yes; N – no; * indicates statistical significance, p<0.05
Figure 39. Graphs A, B and C showing the comparison of plasma PLP, PL and PA between autistic and control populations.

There is no significant difference for PLP (p = 0.30) or PA (p = 0.07). PL is significantly lower in the autistic population (p=0.04) ns = not significant.
Ratios of the three predominant vitamers found in plasma (PLP, PL and PA) were considered as a reflection of PLP flux through the main catabolic pathway.

In Table 35 and Figure 40 B₆ vitamer results are expressed as ratios of PLP: PL and PL: PA. Using unpaired t-tests there were no significant differences in B₆ vitamer ratios between autistic and control populations.

Table 35. B₆ vitamer ratios in autistic and control population showing range, mean, median, standard deviation, standard error, results of Kolmogorov-Smirnov (KS) normality test and p value of unpaired t-test

<table>
<thead>
<tr>
<th></th>
<th>Autism</th>
<th>Control</th>
<th>Autism</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Range</td>
<td>6.8 – 23.9</td>
<td>5.2 – 19.2</td>
<td>0.05 – 0.41</td>
<td>0.09 – 0.7</td>
</tr>
<tr>
<td>Mean</td>
<td>12.0</td>
<td>10.9</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>Median</td>
<td>11.5</td>
<td>10.4</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>Standard</td>
<td>4.2</td>
<td>4.1</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>deviation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard error</td>
<td>0.9</td>
<td>0.9</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Passed KS</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>normality test?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.4</td>
<td></td>
<td>0.85</td>
<td></td>
</tr>
</tbody>
</table>
Figure 40. Comparison of B₆ vitamer ratios (PLP: PL and PL: PA) between autistic and control populations

No significant differences are observed between groups (PLP: PL p = 0.40; PL: PA p = 0.85).
The relationship between plasma PLP and PL and between plasma PL and PA was further investigated using linear regression analysis and correlation co-efficients. Figures 41 and 42 show the positive correlations observed in both autistic and control groups.

![Graph A](image1)

![Graph B](image2)

**Figure 41.** Significant positive correlation between plasma PLP and PL in both autistic (A) and control (B) populations

Autistic: Pearson $r = 0.73$, $r^2 = 0.53$, $p = 0.0002$; Control: Pearson $r = 0.74$, $r^2 = 0.55$ and $p = 0.0001$
Figure 42. Significant positive correlation between plasma PL and PA in the control population (B).

Control: Pearson r = 0.62, \( r^2 = 0.39 \) and p = 0.004**; Autistic: Pearson r = 0.26, \( r^2 = 0.07 \), p = 0.26
5.6.4.1 The relationship between plasma PLP and whole blood serotonin

The relationship between plasma pyridoxal phosphate (PLP) concentration and whole blood serotonin was investigated using linear regression analysis and correlation (Figure 43). There is no relationship between plasma PLP and whole blood serotonin in the autistic or control group (autistic group: Pearson $r = -0.2$, $r^2 = 0.04$, $p=0.38$; control group: Pearson $r = -0.19$, $r^2 = 0.037$, $p=0.39$).

![Figure 43](image)

Figure 43. Relationship of plasma pyridoxal phosphate (PLP) and whole blood serotonin in autistic group (A) and control group (B)

Autism: Pearson $r = -0.2$, $r^2 = 0.04$, $p=0.38$; Control group= Pearson $r = -0.19$, $r^2 = 0.037$, $p=0.39$
The relationship between plasma B₆ concentrations (PLP, PL and PA) and age was also investigated using correlation coefficients and linear regression analysis (Figures 44-46). There is no correlation with age for either group for any of the vitamers. PLP in autism (Pearson r = -0.08, r² = 0.006, p=0.7) and control populations (Spearman r = 0.03, r² = 0.00, p=0.97); PL in autism (Spearman r = 0.07, r² = 0.005, p=0.75) and control population (Pearson r = -0.19, r² = 0.03, p=0.41); PA in autism (Spearman r = 0.25, r² = 0.06, p=0.28) and control subjects (Pearson r = 0.12, r² = 0.015, p=0.6).
Figure 44. Relationship of plasma pyridoxal phosphate (PLP) with age in autism group (A) and control group (B)

Autism: Pearson $r = -0.08$, $r^2 = 0.006$, $p=0.7$; Control: Spearman $r = 0.03$, $r^2 = 0.00$, $p=0.97$
Figure 45. The relationship of plasma pyridoxal (PL) with age in autism group (A) and control group (B)

Autism: Spearman $r = 0.07$, $r^2 = 0.005$, $p=0.75$; Control: Pearson $r = -0.19$, $r^2 = 0.03$, $p=0.41$
Figure 46. The relationship of plasma pyridoxic acid (PA) with age in autism group (A) and control group (B)

Autism: Spearman $r = 0.25$, $r^2 = 0.06$, $p=0.28$; Control: Pearson $r = 0.12$, $r^2 = 0.015$, $p=0.6$
5.6.5 Plasma dopa decarboxylase (DDC) activity in autistic and control populations

Results for plasma DDC activity in the autistic and control cohorts are shown in Table 36. Plasma DDC activity is normally distributed in both populations and passed the Kolmogorov-Smirnov test for normality.

Table 36. Activity of dopa decarboxylase in plasma of autistic and control group showing range, mean, median, standard deviation and standard error

<table>
<thead>
<tr>
<th></th>
<th>Autism</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Range (pmol/min/ml)</td>
<td>14.0 – 72.8</td>
<td>21.0 – 112.5</td>
</tr>
<tr>
<td>Mean (pmol/min/ml)</td>
<td>36.6</td>
<td>57.5</td>
</tr>
<tr>
<td>Median (pmol/min/ml)</td>
<td>32.0</td>
<td>55.6</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>18.3</td>
<td>24.2</td>
</tr>
<tr>
<td>Standard error</td>
<td>3.9</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Statistical comparison of the patient and control groups using an unpaired t-test demonstrated that cases in the autistic population have significantly lower plasma DDC activity than those in the matched control population as shown in Figure 47 (p = 0.003).
Figure 47. Comparison of dopa decarboxylase activity in plasma of autistic subjects compared to a control group

Significantly reduced activity is seen in the autistic population (p = 0.003)

Regression analysis was used to investigate the relationship between plasma DDC activity and whole blood serotonin. In the autistic group, whole blood serotonin shows a positive correlation with DDC activity but this does not reach statistical significance (Pearson r = 0.35, r² = 0.12, p = 0.1). There is no apparent relationship between whole blood serotonin and plasma DDC activity in the control population (Pearson r = 0.05, r² = 0.002, p = 0.85) (Figure 48).
Figure 48. Relationship of whole blood serotonin to dopa decarboxylase activity in plasma of autistic group (A) and control group (B). A positive correlation between whole blood serotonin and dopa decarboxylase activity is seen in the autistic group but this is not statistically significant (Pearson r = 0.35, r² = 0.12; p = 0.1); no relationship is evident in the control group.
Linear regression analysis and correlation was used to investigate the relationship between plasma DDC activity and plasma pyridoxal phosphate (PLP) (Figure 49). In both groups PLP shows a positive correlation with plasma DDC activity which reaches statistical significance in the autistic group (autism group: Pearson r = 0.507; $r^2 = 0.26$; p = 0.016; control group: Pearson r = 0.37; $r^2 = 0.14$; p = 0.09).

Figure 49. The positive correlation of plasma dopa decarboxylase (DDC) activity with plasma pyridoxal phosphate (PLP) in the autistic (A) and control (B) population

The relationship is statistically significant in the autistic group (Pearson r = 0.507; $r^2 = 0.26$; p = 0.016) and not in the control group (Pearson r = 0.37; $r^2 = 0.14$; p = 0.09).
Linear regression analysis and correlation was used to investigate the relationship between plasma DDC activity and age in the autistic and control groups (Figure 50). There is a negative correlation in both groups which is more pronounced in the autistic population but does not reach statistical significance in either group (autism: Pearson $r = -0.35$, $r^2 = 0.12$, $p = 0.11$; controls: Pearson $r = -0.20$, $r^2 = 0.04$, $p = 0.39$).

![Graph A](image1)

**Figure 50.** The relationship between plasma dopa decarboxylase activity and age in the autistic (A) and control (B) group

Autism: Pearson $r = -0.35$, $r^2 = 0.12$, $p = 0.11$, not significant; Controls: Pearson $r = -0.20$, $r^2 = 0.04$, $p = 0.39$, not significant.
5.6.5.1 The relationship of dopa decarboxylase activity and large neutral amino acids in plasma

The concentration of plasma amino acids measured in the autistic and control group are presented in Section 5.6.3.

Figures 51 & 52 and Tables 37 & 38 show the results of linear regression analysis of plasma DDC activity and plasma large neutral amino acids in the autistic and control groups. In the autistic group there is a positive correlation in all cases with highly significant results for tryptophan, tyrosine and phenylalanine. This pattern mirrors the relationship described in the preceding section for whole blood serotonin and large neutral amino acids in plasma. There is no relationship in the control group.

Table 37. Pearson correlation coefficient (r), $r^2$ and p values of linear regression of plasma dopa decarboxylase activity and plasma concentration of large neutral amino acids in autistic population

<table>
<thead>
<tr>
<th>Graph</th>
<th>Amino acid</th>
<th>Pearson r</th>
<th>$r^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tryptophan</td>
<td>0.53</td>
<td>0.28</td>
<td>0.01</td>
</tr>
<tr>
<td>B</td>
<td>Tyrosine</td>
<td>0.70</td>
<td>0.49</td>
<td>0.0004</td>
</tr>
<tr>
<td>C</td>
<td>Phenylalanine</td>
<td>0.55</td>
<td>0.30</td>
<td>0.01</td>
</tr>
<tr>
<td>D</td>
<td>Isoleucine</td>
<td>0.36</td>
<td>0.13</td>
<td>ns</td>
</tr>
<tr>
<td>E</td>
<td>Valine</td>
<td>0.36</td>
<td>0.13</td>
<td>ns</td>
</tr>
<tr>
<td>F</td>
<td>Leucine</td>
<td>0.34</td>
<td>0.12</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant, p>0.05

Table 38. Spearman correlation coefficient (r), $r^2$ and p values of linear regression of plasma dopa decarboxylase activity and plasma concentration of large neutral amino acids in control population

<table>
<thead>
<tr>
<th>Graph</th>
<th>Amino acid</th>
<th>Spearman r</th>
<th>$r^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tryptophan</td>
<td>0.11</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>B</td>
<td>Tyrosine</td>
<td>-0.25</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>C</td>
<td>Phenylalanine</td>
<td>0.03</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>D</td>
<td>Isoleucine</td>
<td>0.07</td>
<td>0.06</td>
<td>ns</td>
</tr>
<tr>
<td>E</td>
<td>Valine</td>
<td>0.22</td>
<td>0.08</td>
<td>ns</td>
</tr>
<tr>
<td>F</td>
<td>Leucine</td>
<td>0.29</td>
<td>0.03</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant, p>0.05
Figure 51. The relationship of plasma dopa decarboxylase (DDC) activity with large neutral amino acids in autistic population

A = tryptophan; B = tyrosine; C = phenylalanine; D = isoleucine; E = valine; F = leucine
Figure 52. The relationship of plasma dopa decarboxylase (DDC) activity with large neutral amino acids in the control population

A = tryptophan; B = tyrosine; C = phenylalanine; D = isoleucine; E = valine; F = leucine
The tryptophan: large neutral amino acid ratio (Trp: LNAA) for the autistic group and control groups are presented in Tables 30 & 31, Section 5.6.3. The Trp: LNAA ratio was compared to whole blood serotonin and plasma dopa decarboxylase activity using correlation co-efficients and linear regression for both populations (Figures 53 & 54). There is no statistically significant relationship evident in either group.

Figure 53. Relationship of whole blood serotonin (A) and plasma dopa decarboxylase (B) with Trp: LNAA

A positive correlation exists between Trp: LNAA and whole blood serotonin (Pearson r = 0.39, $r^2 = 0.15$, p = 0.08); there is no relationship between Trp: LNAA and DDC activity (Pearson r = -0.01, $r^2 = 0.00$, p = 0.96).
Figure 54. The relationship of whole blood serotonin (A) and plasma dopa decarboxylase (B) with Trp: LNAA in the control population.

A negative correlation exists between Trp: LNAA and whole blood serotonin (Spearman r = -0.26, $r^2$=0.09, p=0.4, ns); there is no relationship between Trp: LNAA and DDC activity (Spearman r = -0.159, $r^2$=0.02, p= 0.7, ns).
5.7 **DISCUSSION**

Autism is a complex neurodevelopmental disorder for which the underlying pathophysiology remains elusive. The study presented in this chapter investigated children and young adults with autism and Asperger’s syndrome from a biochemical perspective, in particular the pathway of serotonin biosynthesis, with a view to gaining an improved understanding of potentially aberrant, disease-causing processes. This approach is complementary to genetic studies and may ultimately help to unravel this complex and disabling neuropsychiatric condition.

Autistic subjects were drawn from a tertiary paediatric clinic and, through the use of detailed, validated psychological assessments, are a clinically well-defined group. They form a homogenous high-functioning/Asperger’s phenotype; the vast majority have no other medical or psychiatric co-morbidities and are thus medication free.

Ideally a control group would consist of well-matched, healthy medication-free subjects, however in paediatric research this is ethically challenging and a well matched population in whom the underlying disease process is not known to influence the research outcomes is usually the best acceptable option.

In this study the control group are well matched for age, ethnicity and gender and have a variety of medical diagnoses, making any single confounding factor less likely to affect results. Many of the control subjects with a disorder of cholesterol metabolism have been identified through cascade family screening and attend the clinic for surveillance and lifestyle advice only, thus represent healthy individuals. Those with medium chain acyl CoA dehydrogenase deficiency and α₁-antitrypsin deficiency have no evidence of liver disease and have an unmodified dietary intake. The single control subject with galactosaemia receives a lactose-free diet which is not known to affect serotonin or vitamin B₆ metabolism.

None of the medications taken by a small number of autistic and control subjects are thought to influence B₆ or serotonin metabolism.
5.7.1 Whole blood serotonin

Results from this study show no significant difference in serotonin concentration between autistic and control groups. This is in contrast to much of the previously published literature which has demonstrated hyperserotonemia in up to one third of subjects with autistic spectrum disorder. Reasons for this apparent discrepancy will be considered.

The explanation for lack of difference between our control and autistic population appears to lie in the absolute values of serotonin for the autism group which has a lower mean serotonin concentration than in previously published work. In contrast, the group mean for our control population is comparable with previous published data (229). On initial inspection a large number of subjects in the autism group have serotonin concentrations which fall above the upper limit of our laboratory reference range (1600 nmol/L), however a comparison of proportions lying over this limit between the autism and control population (using Fisher’s exact test) demonstrate that this not does differ between the two groups. Overall these findings suggest that our laboratory upper limit of normal may be invalid for the paediatric population and emphasises the importance of collecting data in a well matched control population.

Correct sample handling is important for accurate measurement of serotonin as the metabolite deteriorates quickly if left at room temperature. In this study all samples were frozen at the bedside by the researcher according to protocol and analysed in a single laboratory using a validated method therefore it is unlikely that sample handling or analysis is a source of error. Previous work has suggested that the time of sampling may affect serotonin concentration (352). All samples in this study were collected at a similar time of day; it was impractical to control for season of collection in this study.

Various factors which may influence serotonin concentrations have been studied in relation to individuals with autism. Detailed work by McBride et al. (359) suggested that elevated serotonin was most pronounced in pre-pubertal autistic children and that ethnicity strongly influenced serotonin concentration with Black and Latino youngsters having higher levels than those seen in White subjects. Along with others (229) they were also able to show that non-autistic mental retardation was not
associated with hyperserotonaeimia. In concluding, this group suggested that hyperserotonaeimia in autism may have been previously overestimated due to poor matching of control groups. Subjects of the study reported here are well matched for age, gender and ethnicity. The average age of our autistic group (12 years 1 month) however, was higher than that of the pre-pubertal children reported by McBride *et al.* and may partly explain the lower mean serotonin that we observed. The observation that serotonin correlates negatively with age in the ASD population in this work (Figure 5A) adds support to the consideration that serotonin, which may show a pronounced elevation in early childhood, gradually reduces to normal levels with age in autistic subjects. Interestingly the youngest autistic patient in this study also had the highest serotonin concentration.

A subsequent, larger study by Mulder *et al.* (229) later showed no effect of a range of clinical and demographic variables including age and pubertal status, upon serotonin concentration. However, possibly because of the large numbers, they were able to demonstrate a bimodal distribution of serotonin concentration in autism and Pervasive Developmental Disorder – Not Otherwise Specified (PDD-NOS), with over one third of individuals falling into the hyperserotonaeimic subgroup. Surprisingly, this biochemically distinct group of subjects could not be distinguished from normoserotonaeimic subjects by any clinical / psychological variable, including an extensive number of behavioural assessments.

Only a few other studies have explored the relationship of serotonin to clinical phenotype, however those that have, also failed to find any relationship between elevated serotonin and clinical functioning (360;361). The results presented here replicate these findings in that no relationship with serotonin was evident for any of the multiple clinical variables measured and suggest that hyperserotonaeimia is not directly related to clinical disease expression (data analysed by Prof Skuse, Department of Neurosciences and Mental Health, Institute of Child Health; data not shown). Although superficially promising, it appears that serotonin is not a useful biomarker for the diagnosis of autism for all age groups and all degrees of clinical severity.

A genome-wide screen in almost 600 members of a single large pedigree (not known to suffer with autism) has recently identified β3 integrin (*ITGB3*) as a quantitative
trait locus (QTL) determining whole blood serotonin (362). Subsequent studies have shown that a coding variant of \textit{ITGB3} is associated with autism susceptibility which appears to have a different effect in males compared to females. It is interesting to consider whether the presence of this coding variant would predict the elevated serotonin in our group where no other measured clinical or biochemical variable is able to. This protein is particularly interesting because the integrin class of cell adhesion molecules have recently been found to play a role in the regulation of synaptic plasticity and in some cases may be disrupted during development resulting in autism (363;364).

As a group, patients with autistic spectrum disorders share common features of impaired reciprocal interaction, impaired communication and restricted patterns of behaviour however within this, the phenotype is diverse. Individuals with Asperger’s syndrome represent one end of the autistic spectrum and are often described as ‘high-functioning’ autism. In contrast to others under the autistic umbrella, this group have no delay or retardation in language development (although use of language may be altered) and normal or high intelligence. Diagnostically these children and adults are more challenging and they often go undiagnosed. It is maybe not surprising, therefore, that only two groups have examined serotonin in a small number of Asperger’s syndrome patients (229;365). In both studies only five patients were included and similar to the results presented here, no mean elevation was seen compared to control populations.

\textbf{5.7.2 Plasma B\textsubscript{6} vitamers}

An interest in the use of mega-dose vitamin therapy for psychiatric disorders began in the 1950’s with the treatment of schizophrenic patients and, subsequently, many studies have explored the therapeutic use of vitamin B\textsubscript{6} in autism. In some patients high dose pyridoxine was noted to be associated with side effects such as irritability and hyperacusis; symptoms which could be ameliorated with magnesium, thus later work investigated combined vitamin B\textsubscript{6} -magnesium treatment, some of which reported an improvement in the speech, language and social functioning of patients (366).
Using strict inclusion criteria, a Cochrane review (366) was only able to include three out of nineteen such studies investigating the efficacy of combined B₆ and magnesium in the treatment of social, communication and behavioural responses of patients with autistic spectrum disorder. Due to small sample sizes the review was unable to reach any firm conclusions, thus additional better designed trials are required to answer this important question.

Although not directly related, it is interesting to note the improvement in expressive language and autistic features observed in a small number of children with antiquitin deficiency following an increase in their pyridoxine treatment dose (113;367). This observation has led authors to consider that vitamin B₆ may have a specific effect on speech and language development.

Very few studies have examined plasma levels of vitamin B₆ (in any form) in autistic subjects and those published show conflicting results. Sankar (368) used a yeast method to measure ‘pyridoxine’ in ‘severely disturbed children’ 19 of whom were described as autistic. He found no difference in the concentration of pyridoxine in autism compared to other mental health disorders or normal controls. More recently Adams et al. (369) found total plasma B₆ (PL, PM, PN and their phosphorylated forms) to be elevated in 11 autistic subjects compared to matched control subjects. Despite elevated total B₆, plasma pyridoxal phosphate levels were actually reduced in the autism group leading the authors to conclude that pyridoxal kinase activity may be defective. This study used a dated protozoological method (328) to measure B₆ that has since been superseded by other more advanced techniques such as tandem mass spectrometry. Both of the studies discussed have flaws including poor clinical characterisation of subjects and small group numbers, alongside the problems with methodology.

Given the important role of PLP as a cofactor for AADC in serotonin synthesis and the unresolved question of B₆ treatment efficacy and its mechanism in children with autism, plasma B₆ vitamers were measured in this study.

In agreement with the work of Sankar (368) this study found no statistically significant difference in total plasma vitamin B₆ concentration between autistic and control groups. The autistic group in fact had a lower mean concentration than
controls for each of the most predominant vitamers (PLP, PL and PA) although this difference was statistically significant for PL only. Considering the pathway for PLP catabolism (Figure 4), there is no obvious physiological explanation for a reduction in PL in the absence of any perturbation in PLP or PA. This is supported by the observation that the ratios of PLP: PL and PL: PA do not differ between groups.

Of the six individuals with the highest total plasma B<sub>6</sub> concentration in the control group, three were taking a statin medication for familial hypercholesterolaemia, one low dose amlodipine for hypertension and two were medication free. There is no obvious mechanism for statin treatment to cause an elevation of plasma B<sub>6</sub> however the fact that several subjects in the control group as a whole have familial hypercholesterolaemia and part of their clinical management involves advice about healthy lifestyle, including diet, may be relevant. Recommendations for a healthy diet include advice to maximise intake of fruit, vegetables and lean meat which contain generous amounts of vitamin B<sub>6</sub>.

Thus the best interpretation of these results as a whole is that the control group have a marginally higher total B<sub>6</sub> concentration than the autism group due to their dietary intake. An accurate assessment of dietary intake would help to resolve this question; however the food diary was not completed or returned in a sufficient number of subjects in this study to make a comparison of B<sub>6</sub> dietary intake possible. The finding that neither the mean plasma concentration of PLP in the autism group, nor the plasma concentration in any individual within the group, fell below the laboratory reference range is in keeping with this conclusion; it is not necessary to look for causes of sub-physiological values in autism.

Individual plasma B<sub>6</sub> vitamer concentrations in the paediatric population have not been published previously and only a few studies measure all the vitamers by mass spectrometry in older age groups. Results presented here [mean age 12 years 7 months] are within a similar range to those for adult populations [mean age 57 years] (301) although it is notable that the mean concentrations for PLP and PA are higher in our paediatric group. Many metabolites, including the CSF and plasma concentration of PLP, reduce with age so this observation is not unexpected (95;98). In keeping with results from adult populations PN, PM and PNP were either not detectable or present in very small amounts. PMP was present in low amounts in
some subjects studied here, but not in any of the adult population studied by others previously (301). The finding that none of the three predominant vitamer concentrations (PLP, PL or PA) change with age in our autism and control group probably reflects the relatively narrow age range studied, as all of the subjects fall within the ‘childhood’ bracket.

Although PLP acts as cofactor for AADC, this decarboxylation reaction is not usually the rate limiting step for serotonin synthesis, thus it is not surprising that no relationship is evident between PLP and serotonin concentration in either the autistic or control group. Similarly, within the physiological range, PLP concentration is not seen to effect levels of 5-HIAA in CSF (unpublished observation). In the severe PLP deficiency seen in PNPO deficient patients however, turnover of serotonin (and dopamine) in the CNS is significantly reduced as evidenced by reduced 5-HIAA (and HVA) concentration in CSF (123).

Although no differences were observed between autistic and control groups overall, this work has answered important questions; if vitamin B₆ is indeed of benefit in the treatment of autism, it is unlikely to be due to B₆ deficiency per se. Whether increasing plasma PLP concentrations above the normal range by pharmacological means could improve autistic traits by increasing DDC activity (as evidenced by positive correlation between PLP and DDC, Figure 18A) is not known.

5.7.3 Plasma dopa decarboxylase (DDC) activity

Plasma DDC activity has not previously been reported in autistic spectrum disorder and in the group reported here it is significantly reduced compared to an age matched population. In this study, DDC activity appears to be a more reliable indicator of autistic spectrum disorder than serotonin and this may provide an important insight into underlying disease mechanisms.

The mean and range of DDC activity measured in our control group is comparable to that previously reported in paediatric populations (288;307;358) and of note is higher than that seen in control adult populations. We found a weak negative correlation of DDC activity with age in the both the autistic and control paediatric populations;
similarly Verbeek et al. (358) showed that AADC activity decreased slightly with age. Not surprisingly, plasma PLP concentration correlates positively with AADC activity in both groups and reaches statistical significance in the autistic population.

The laboratory method used in this study for measurement of DDC activity in plasma was first described by Boomsma and Schalekamp (370). Subsequently it has been adapted and used for diagnostic confirmation in the first description of genetic AADC deficiency (288). The method continues to be used for the diagnosis of such patients alongside mutational analysis of the gene.

It is of note that the procedure differs from other enzyme assays in that prior to addition of the substrate L-dopa, a long incubation time of 2 hours with the cofactor PLP in the presence of dithioerythritol (DTE) is required. Boomsma and Schalekamp (370) explore possible reasons for this in their work and postulate that the requirement for additional exogenous PLP may be two-fold; partly to protect the enzyme-bound PLP against Schiff base formation with other amino acids e.g. tyrosine and partly to overcome the reversal of enzyme activity by side reactions with PMP. The fact that greatly increased enzyme activity was found after prolonged incubation of plasma with DTE in addition to PLP supports the hypothesis that DTE protects the free sulphhydril group of the enzyme thus allowing more cofactor binding. The group also suggested that DTE may prevent Pictet-Spengler adducts forming between PLP and dopamine, thus preventing conversion of formed dopamine to a tetrahydroiso-quinolone derivative (370). Assay of AADC in this study took account of these observations, thus maximising capacity of the enzyme to convert L-dopa to dopamine in both autistic and control groups.

Secondary factors that may lead to reduced AADC activity measured using this assay also warrant consideration. In vitro work shows that the activity of plasma AADC measured as conversion of L-dopa into dopamine is moderately inhibited by 5-HIAA but not by serotonin, HVA or 3-methoxytyrosine (358). In our patient samples it is unlikely (although not impossible) that a high 5-HIAA concentration is contributing to reduced AADC activity. 5-HIAA is a stable metabolite, but it should not be present in greater concentrations in the autistic group compared to the control group because serotonin concentration did not differ between the two groups. Additionally, in support of this, a small number of published studies show no difference in urinary
or CSF 5-HIAA in autism compared to control groups (371;372). 5-HTP shows a strong inhibitory effect on the conversion of L-dopa to dopamine \textit{in vitro} (358) which may be contributing to the low AADC activity in the autistic group and is discussed below.

Although their physiological significance is unknown, other studies have suggested that AADC can undergo an irreversible inactivation through permanent incorporation of both serotonin and PLP into its structure (373). Given the observation of elevated serotonin in some children with autism, this reaction should be considered as a possible cause of reduced AADC activity, however in our study no correlation was seen between AADC and serotonin and importantly hyperserotonemia was not present.

Cofactor availability is crucial for AADC activity as exemplified by PNPO deficient patients where significant CSF monoamine depletion may be observed (95;307). In the enzyme assay described here PLP is present in excess, thus cofactor availability should not affect AADC activity; however other metabolites such as PMP may compete for the cofactor binding site. No difference in plasma PMP concentration was observed between autism and control groups and the concentration was not elevated in either group, thus it is unlikely to be contributing to reduced AADC activity in this study. It should also be considered that, given its reactivity, PLP may form an adduct with an unidentified metabolite in the plasma of ASD patients (as is observed in Antiquitin deficiency and Hyperprolinaemia II, Figures 5 and 6), thus making it unavailable for its role as a cofactor. However there is no evidence of such a process happening \textit{in vivo}, as there is no evidence of PLP deficiency either clinically or on biochemical investigation.

The AADC protein is known to contain several motifs which serve as recognition sites for phosphorylation enzymes (cAMP-dependent protein kinase, protein kinase-C, calcium calmodulin dependent protein kinase II and proline directed protein kinase), the activity of which result in an increase in AADC activity (186). This mechanism of phosphorylation probably underlies the increase in AADC activity seen following alterations in dopamine receptor activation (186); further work would be necessary to exclude hypo or hyper-phosphorylation as a cause of altered AADC activity in our samples.
Further consideration of the cause of reduced AADC activity must include the possibility of a genetic deficit. Mutations in the gene encoding AADC result in a clinical phenotype very different to that of the children in the described disease group, that is characterised by severe hypotonia, occulogyric crises, delayed development and autonomic symptoms such as excessive sweating and temperature instability (374). This autosomal recessive disorder was first recognised in twins found to have a severe deficiency in the monoamine neurotransmitters dopamine and serotonin (375) and analysis of CSF concentrations of neurotransmitter amine metabolites remains important in the diagnosis of this condition alongside measurement of plasma AADC activity and mutational analysis of the AADC gene. Recently, Brun et al. (374) reviewed 24 different mutations that have been described in AADC deficient patients; a substitution mutation in intron 6 affecting splicing was found to be the most common with an allele frequency of 45% in patients and although the majority of mutations lie in the coding regions, one has been described in the non-coding 5’UTR. Unfortunately the prognosis for many children with this condition is poor, with treatment options such as dopamine agonists, pyridoxine and MAO inhibitors having limited effect (374).

Disorders of language and social communication have not been described in AADC deficient patients, possibly because other difficulties predominate, however there is evidence of an increased burden of neuropsychiatric disease in the first degree relatives and extended family of AADC patients (376). Although ASD and Asperger’s syndrome per se do not feature in the pedigrees of AADC deficient patients (possibly due to under-diagnosis), bipolar disorder, anxiety, attention deficit-hyperactivity disorder and depression have all been reported. Additionally, symptoms of anxiety and depression are not uncommon in ASD patients and mental health disorders such as schizophrenia, depression and obsessive-compulsive disorder are more common in parents of autistic children (377;378). As neuropsychiatric diseases are increasingly considered to lie on a continuum sharing underlying pathogenic processes, it is not inconceivable that reduced activity of DDC may be responsible for, or at least involved in, some of these common features and suggests that the findings of this study are of relevance to the clinical picture.
Although plasma activity of DDC in ASD (mean activity 36.6 pmol/min/ml) was reduced compared to the normal paediatric population (57.5 pmol/min/ml) in this study, it is not as low as that described in AADC deficient patients who have extremely low (<10 pmol/min/ml) or undetectable activity in plasma (351;358). The activity levels are however similar to those reported in heterozygote carriers of a mutation in the AADC gene which is of interest in the context of the mental health disorders described above.

Given its central role in monoamine biosynthesis, the search for AADC mutations in relation to neuropsychiatric disease has been the focus of several studies. Anderson et al. (379) specifically examined the association of genes in the serotonin system to autism but found no evidence of linkage to any specific gene, including AADC. A subsequent study which examined a larger cohort of autistic individuals did however find a significant association between an intronic marker (rs6592961) in the AADC gene and autism. The group suggest this may be one of many common susceptibility alleles likely to have modest effect (380).

Two frequent sequence variants in the non-coding region have been identified in patients with bipolar disorder; a 1 base pair deletion in the promoter and a 4 base pair deletion in the untranslated exon 1 (381;382). How these changes relate to the disease process or affect gene function is not known.

In summary, it seems unlikely that a mutation in the coding region of the AADC gene is responsible for the findings of low enzyme activity described in this study. It is however theoretically possible, that factors controlling the expression of AADC specifically within serotonergic neurones, possibly during early development, may play a role and is discussed below.

In man, the AADC gene is over 85 kb in length and comprises 15 exons. It resides on chromosome 7p21.1 – p12.3. Several splice variants have been identified in human and animal tissues which show differences in the coding and non-coding regions.

Two of the AADC mRNA variants differ only in their 5’untranslated region (5’UTR) and code an identical amino acid sequence of 480 amino acids with a molecular mass.
of 53.9kDa (382). The different 5’UTR regions are encoded by two distinct exons which undergo alternative splicing and direct tissue expression of AADC. They have been termed ‘neuronal type’, (N1) and ‘non-neuronal type’, (L1). Differential regulation of AADC expression in neuronal and non-neuronal tissues raises the possibility of differing functions in these tissues which is incompletely understood (383;384).

Work by O’Malley et al. (384) and Chang et al. (385) showed that AADC can also undergo alternative splicing of exon 3 which generates two different protein isoforms; AADC$_{480}$ and AADC$_{442}$. Both transcripts are widely expressed with AADC$_{442}$ predominating in neuronal and most non-neuronal tissues. The highest level of both transcripts is seen in the liver, where AADC$_{480}$ is the most abundant of the two. The sequence of amino acids deleted in AADC$_{442}$ (exon 3, amino acids 68 -105) lies in one of the most well conserved regions of the AADC protein, suggesting they are of particular importance in the enzyme structure or function. Given that, of the 2 transcripts, only AADC$_{480}$ shows activity towards L-dopa and 5HTP as a substrate, it is likely that exon 3 is involved in substrate recognition and binding. Little is known of the role of AADC$_{442}$ however it may be involved in decarboxylation of other substrates, for example phenylalanine or L-tyrosine (384).

Further evidence that the AADC gene undergoes complex processing resulting in multiple mRNA forms comes from work of Vassilacopoulou et al. (2004) (386) who report another splice variant of AADC. This alternative variant (alt-AADC) which lacks exons 10 - 15 and includes an alternative exon 10 was found to be highly expressed in human kidney. Little more is known about this form and further studies are required to understand the protein function and enzymatic activity of this variant.

Using the method described previously, plasma AADC activity was found to be significantly reduced in two PNPO deficient patients, despite the addition of a saturating concentration of PLP. Work in neuroblastoma cell culture also showed AADC activity in B$_6$ deficient media was reduced by 70% compared to controls (307). Here the authors highlighted that PLP has additional functions beyond its role as cofactor to AADC and suggest that PLP may act interact with transcription factors to affect AADC expression (307). Other factors known to affect AADC mRNA levels include dexamethasone, Interleukin 1β (I1β), prostaglandin E2 (PGE2) which,
amongst others, increase AADC mRNA and vigabatrin and amphetamine all of which decrease AADC mRNA. Effects of these factors on specific tissues are unknown.

A reduction in the expression of neuronal AADC resulting in reduced plasma AADC activity could feasibly be due to an inherited mutation in the AADC promoter regions or in the genes encoding the transcription factors which act upon these regulatory regions. Neurone-specific gene expression in vertebrates is controlled by a complex interplay of neurone-specific enhancer elements, and of silencers which suppress promoter activity in inappropriate non-neuronal cells (387). A neurone-specific AADC promoter region containing three positive regulatory elements and two negative regulatory elements was first described by Le Van Thai et al. (388). Subsequently two factors were identified which appear to inhibit expression of the neuronal form (leukaemia inhibitory factor, LIF and ciliary neurotrophic factor, CNTF) and a non-neuronal promoter was also identified (387). Further work unravelling the complexity of AADC gene expression via transcription factors acting at these promoter sites has shown an interplay of winged helix/forkhead (hepatocyte nuclear factor 3) and POU-domain (brn-2/N-oct-3) transcription factors (389).

As a neurotransmitter, serotonin has many diverse functions in the central nervous system, both during early development and in adult life. It is considered likely that abnormalities in the serotonin system are involved to some degree in the development of social and communication disorders (234;390). In part this stems from the observation that some children with autism show an improvement with selective serotonin reuptake inhibitors (SSRI) and also that tryptophan depletion (resulting in reduced central production of serotonin) exacerbates autistic symptomatology. Perhaps most convincing is work by Chugani et al. (230;231) who use positron emission tomography (PET) imaging of a tryptophan analogue to show developmental differences in brain serotonin synthesis in autistic children.

To understand how serotonin, AADC and the development of autism may be linked it is helpful to consider the development of serotonergic neurones and work in animal models offers interesting insights into normal human development. Amongst many transcription factors, one ETS domain factor, Pet-1, is of particular interest as it
appears to be an essential element of the transcriptional program that specifically triggers central serotonin neuron differentiation early in rat embryonic life (391). Its specific developmental expression in the hindbrain region begins before the appearance of serotonin and continues into adult life, strongly suggesting a function to establish and maintain the serotonergic phenotype. It achieves specificity for serotonergic differentiation by binding to specific sites in the promoter regions of genes that define the serotonergic neuron i.e. tryptophan hydroxylase, serotonin transporter (SERT), vesicular monoamine transporter (VMAT) and AADC (391). A PEA-3 like binding domain for Pet-1 has been identified in intron 1 of the AADC gene (391;392) which, if mutated, could lead to an isolated deficiency of AADC and hence serotonin, within serotonergic neurones at a crucial developmental stage and perhaps with ongoing consequences into later life. Given the plasticity of the developing nervous system it is likely that compensatory mechanisms would modulate the phenotype to differing degrees in individuals. Mutations within the Pet-1 gene itself or its specific binding sites on other serotonergic gene promoters may also produce a similar spectrum of clinical problems relating to serotonin deficiency. How this proposed mechanism may explain the findings of reduced plasma AADC activity is further discussed below.

AADC protein is a homodimer consisting of two monomers, each of 480 amino acids. The crystal structure suggests that each monomer has three domains; a large central domain, a C-terminal small domain and an N-terminal domain (183). The enzyme active site lies close to the interface of the monomers at the large central domain. Each homodimer binds two molecules of pyridoxal phosphate at lysine residue 303 within the active site, forming an internal aldimine through a Schiff base linkage, as is the case for many PLP dependent enzyme reactions. The PLP – lysine interaction undergoes a transaldimination reaction with the substrate forming a Schiff base between PLP and the substrate. The external aldimine then leaves the amino group of lysine as a free base (184).

Despite conjecture over many years, it is now accepted that AADC activity is the product of a single gene and can catalyse the decarboxylation of several different substrates (185). In vitro, substrate specificity of the enzyme appears to be affected by pH, temperature and relative substrate and cofactor concentration (393). The
differential response to depletion of the cofactor PLP (as a result of a pyridoxine-deficient diet) is interesting, in that a very significant reduction in serotonin production was observed in the brain of PN deficient rats while no change was seen for brain catecholamines in the same experiment (394). \textit{In vivo}, anatomical localisation within dopaminergic versus serotonergic neurones and the consequent availability of substrate is likely to play a major role in the relative production of monoamines by the enzyme.

As its name suggests, in addition to L-dopa and 5-HTP, AADC can catalyse the decarboxylation of all L-aromatic amino acids including L-tryptophan, L-tyrosine and L-phenylalanine (395). Despite the high $K_m$ observed with these substrates, the reactions do appear to proceed \textit{in vivo} as evidenced by the presence of the trace amines in various brain regions, albeit at low concentrations (396-398). The precise physiological role of these substances is not known, however they are thought to play a role in neuromodulation of monoamine metabolism (399).

AADC is localised in various brain regions and peripheral tissues. Centrally it is present within serotonergic, dopaminergic and noradrenergic neurones which, cumulatively, project widely to a large number of brain structures (185). AADC is also expressed in D-cells which are a small group of non-monoaminergic cells located in hypothalamus, striatum, forebrain and cortex (400). It has been suggested that D-cells may be involved in trace amine production, hence the presence of AADC (401).

AADC is also widely expressed in the periphery; predominantly in kidney, liver, enterochromaffin cells of the gut and the adrenal medulla. Its role in some of these locations is apparent, for example in the gut it is involved in serotonin biosynthesis and in the adrenal and kidney it catalyses dopamine formation. Its function in the liver however, where it is particularly abundant, is less clear as biosynthesis of monoamines does not occur here.

Similarly the presence and function of AADC in plasma is not easily explained and indeed the origin of plasma AADC is not known. Boomsma and Schalekamp (402) describe significantly elevated plasma AADC activity in patients with neuroblastoma suggesting that plasma AADC may be of sympathetic nervous system origin.
Considering the findings described in this chapter, the origin of plasma AADC is of importance to understand how, if at all, it may be involved in the pathology of autistic spectrum disorder. If indeed it reflects ‘neuronal’ AADC and its expression is directed by the neuronal form of mRNA then activity of the enzyme observed in plasma may parallel activity within the central nervous system.

Following this hypothesis, it may be expected that AADC activity is decreased in the central nervous system, resulting in a deficiency of serotonin possibly in regions of the brain which are particularly important for social development during \textit{in utero} neurodevelopment. A possible mechanism involving disruption of the complex interplay of transcription factors which precisely drive the development of serotonin containing neurones has been discussed above. Within this framework, if it is considered that neuronal AADC expression and activity is affected in serotonergic neurones only, then the observation that central dopamine production is not significantly affected in autism can be understood.

The apparent paradox of elevated whole blood serotonin in some autistics is not at first easy to explain. One possibility is that if AADC activity is reduced in central serotonergic neurones, then 5-HTP would accumulate proximal to the block. This compound is able to cross the blood brain barrier and would likely spill over into the circulation. As AADC is not saturated at physiological concentrations, increased substrate may result in increased serotonin production in the peripheral circulation via AADC present in another anatomical location for example. The vast majority of whole blood serotonin is contained within platelets which acquire serotonin produced in enterochromaffin cells in the gut which may be the non-neuronal source of elevated serotonin. The absence of a significant correlation of serotonin with plasma AADC activity in controls or autistic subjects in our study is in keeping with this. Elevated concentrations of 5-HTP in the central nervous system may have other manifestations including the production of metabolites lying on the kynurenine pathway (5-hydroxyformylkynurenine and 5-hydroxykynurenine) the physiological and pathological effects of which are unknown.
5.7.4 Amino acids

Abnormalities in plasma amino acids have received little attention in autism research and existing studies often show differing results. There are no striking differences between the autistic and control populations reported here although the small number of samples available for control subjects does limit conclusions. When comparing groups, both plasma glycine and taurine were found to be elevated in autism relative to control patients, the biological significance of this however is unclear as the group mean for both is within the laboratory reference range. Investigation of individual patients may help delineate this further.

Taurine is one the most abundant amino acids in man; in the brain it occupies by quantity the second place after glutamate (403;404). It is involved in several important physiological functions within the central nervous system; as a neuromodulator, a neurotransmitter and a neuroprotector against L-glutamate induced toxicity (404). Its potential role in autistic spectrum disorder is therefore of interest and previous studies have documented evidence of taurine elevation in the plasma and urine of autistic subjects compared to controls (405;406). This has not been a consistent finding however as other groups have described reduced taurine concentrations in plasma (407).

Similarly, due to its CNS actions, glycine may be of importance in the pathophysiology of ASD. As the smallest of the amino acids in man it is widely recognised as a major inhibitory neurotransmitter through its actions upon the glycine receptor (408). Interestingly, it also acts as an activating ligand at the N-methyl-D-aspartate (NMDA) ion channel receptor. To date no abnormalities have been described in the plasma glycine concentration of autistic individuals.

Caution is required in the interpretation of plasma and urinary amino acid concentrations in disease compared to control groups as there are many potential confounding factors including subject age, time of sampling and dietary intake in addition to the well documented difficulties pertinent to autism research of clear phenotype definition.
Ideally plasma amino acid concentrations should be analysed in fasting samples. This was impractical for the current study, however overnight fasting samples were available for some and all other samples were pre-prandial.

As the amino acid precursor of serotonin, consideration of plasma tryptophan concentration is of importance. Tryptophan hydroxylase is not fully saturated at physiological concentrations, thus tryptophan concentration can affect serotonin production (188). CNS availability of tryptophan for serotonin synthesis is dependent not only on tryptophan concentration in the plasma but also on the ratio of tryptophan to the other large neutral amino acids (LNAA) [tyrosine, phenylalanine, valine, leucine and isoleucine] with which it competes for transport into the brain via a transporter, LAT1 (409;410). If some cases of autism are considered as a condition of central serotonin depletion (350) then reduced availability of tryptophan may play a role.

Unlike other amino acids, circulating tryptophan is specifically bound to albumin. The small fraction of ‘free’ tryptophan is thought to be the biologically active form which is available in the circulation for use by tissues and organs, including the brain (411). Results for plasma tryptophan presented here represent total tryptophan concentration as the laboratory method employed breaks tryptophan protein bonds through the use of sulphosalicylic acid prior to measurement.

In this study there is no difference in plasma tryptophan, total LNAA or the Trp:LNAA ratio between autistic and control subjects. Plasma tryptophan is within the laboratory reference range for all autistic subjects except one in whom tryptophan consistently fell well below the normal range. This patient is considered in detail in Chapter 6. One previous study describes significantly lower urinary tryptophan measured by gas chromatography/mass spectrometry in 33 autistic children compared to controls. Lack of information regarding detailed dietary intake and relation to plasma concentrations makes this work difficult to interpret (412).

A study by D’Eufemia et al. (413) demonstrated a significantly lower serum tryptophan to large neutral amino acid ratio (Trp: LNAA) in 40 children with ‘infantile autism’ compared to matched controls. The mean Trp: LNAA ratio for the autistic group in our study (0.12) is higher than that reported (0.087) by D’Eufemia.
et al. (1995) (413) and is more comparable with their control range (0.110). Although the mean age of autistic subjects is similar in both studies, the group of children investigated by D’Eufemia et al. have a mean IQ of 68.1 which is considerably lower than the group presented here and may partly account for the differences.

The relationship between serotonin and dopa decarboxylase activity with the large neutral amino acids was investigated in the autistic and control groups. A significant positive correlation was observed between tryptophan, tyrosine and phenylalanine concentration for both serotonin and DDC in the autistic patients but not the controls (Tables 32 & 33 and Figures 36 & 37; Tables 37 & 38 and Figures 51 & 52).

Discussing serotonin first, if we consider that autistic patients have an accumulation of 5-HTP in the brain as a result of reduced AADC activity (as discussed in Section 5.7.3) then this will leave the central nervous system via the LAT1. This transporter is likely to exhibit a counter flux phenomenon so that the higher the level of plasma LNAA the greater the export of 5-HTP from the brain. The resultant increased concentration of plasma 5-HTP could explain the elevation in whole blood serotonin evident in some autistic children and in others, where the concentration of 5-HTP is not high enough to alter serotonin production, the only abnormality evident is the dependence of serotonin on plasma concentrations of LNAA. The correlation with plasma tryptophan and serotonin is particularly strong which is to be expected as tryptophan is the immediate precursor of 5HTP. Reduced activity of plasma DDC in vitro would be unsurprising in this scenario as 5-HTP strongly inhibits the decarboxylation of L-dopa to dopamine, even when the substrate is present at high concentrations as used in the assay presented here (358). In keeping with this hypothesis where there is a net export of 5-HTP out of the brain in autism and a net import in normal subjects, it has been observed that 5-HTP given to autistic subjects leads to an increase in blood serotonin whereas in controls it does not (414).

The correlation of Tyr, Phe and Trp with DDC activity may perhaps be understood on the basis that these three amino acids compete with 5-HTP for binding to plasma AADC; the greater the concentration of Phe, Tyr and Trp, the less inhibition of AADC by 5-HTP in vitro.
Two recent studies have shown different amino acid abnormalities measured in platelet-poor plasma (platelet depletion in the laboratory after sampling), in small numbers of children with autistic spectrum disorders compared to controls. Tirouvanziam et al. (415) demonstrated reduced levels of polar neutral amino acids and leucine with developmental profiles that were altered compared to controls. Shimmura et al. (416) examined 25 children with high-functioning autism and found higher levels of glutamate and lower levels of glutamine than a matched control group and postulate that these findings may indicate glutamatergic abnormalities in the brain. No such findings were evident in the results reported here, however amino acids were not measured in platelet-poor plasma.

5.8 SUMMARY

In summary, altered serotonin metabolism appears to play a role in the pathology of autism as evidenced by clinical, radiological and biochemical observations. Plasma DDC activity is reduced in some autistic subjects; if this is of neuronal origin it may reflect reduced central serotonin synthesis. One explanation for the decreased DDC activity observed is the selective loss of AADC expression in serotonergic neurones and this may result from reduced action of one or more AADC-targeted transcription factors (e.g. Pet-1) in the developing serotonergic neurones.

Future work to investigate this hypothesis further could include:

1 – Determination of the amount of AADC protein and any post-translational modifications in plasma of ASD subjects compared to controls using protein mass spectrometry

2 – Sequencing of genes encoding transcription factors for serotonergic neurones and their specific binding sites in AADC promoter regions

3 – Further investigation of the biochemistry in autistic subjects by measuring levels of 5-HTP in relation to whole blood serotonin and both 5-HTP and L-dopa decarboxylase activity in the plasma of autistic subjects and measuring AADC activity, 5-HTP and 5-HIAA concentrations in CSF.
Chapter 6
A syndrome of pyridoxal 5’-phosphate, tryptophan and serotonin deficiency associated with epilepsy, ataxia and Asperger’s syndrome; a case study
6.1 INTRODUCTION

Patient DN (not the patients’ real initials) presented with epilepsy, ataxia and Asperger’s syndrome and was referred to the neurometabolic clinic for further investigation of a reduced CSF concentration of pyridoxal phosphate (PLP). Her clinical phenotype was not typical of the previously described inborn errors of B₆ metabolism associated with reduced cerebrospinal fluid PLP and consistent with this, investigations excluded antiquitin and pyridoxamine 5’phosphate oxidase deficiencies.

In addition to a reduced CSF pyridoxal phosphate, her investigations also showed a significantly reduced plasma tryptophan (Trp) concentration. This finding is not commonly encountered in clinical practice and Hartnup disease is the only well described disorder associated with a reduced plasma tryptophan. Hartnup is an autosomal recessive disease which results in deficiency of a neutral amino acid transporter at the epithelial surface of intestinal and renal tubular cells (417). This was excluded in Patient DN as her urinary amino acid profile did not show the striking neutral hyperaminoaciduria characteristic of this condition; additionally her clinical symptoms were very different to those described in Hartnup disease.

In the absence of any other unifying diagnosis, a hypothesis is proposed whereby the primary abnormality is increased activity of tryptophan 2, 3-dioxygenase (TDO) with increased production of metabolites on the kynurenine pathway. TDO is the main enzyme regulating plasma Trp concentration; increased activity lowers plasma tryptophan and diverts Trp away from indolamine synthesis into the kynurenine pathway (173) (Figure 55). The fact that DN had a low whole blood serotonin was in keeping with such a diversion of Trp metabolism caused by increased TDO activity. Increased activity of TDO can be expected to result in elevated kynurenine pathway metabolites and several of these compounds are neurologically active having deleterious consequences in animal models (418). They could therefore be contributing to the observed neurological dysfunction. In addition, kynurenine pathway metabolites have been shown to inhibit pyridoxal kinase (419;420), thus providing a possible explanation for the low CSF PLP and the improvement of some of DN’s neurological problems with B₆ treatment.
The following chapter presents the clinical case history of patient DN, her extended laboratory investigations including sequencing of the TDO2 gene and a further discussion of the possible underlying disease mechanisms and how the proposed hypothesis could be tested.
Figure 55. The kynurenine pathway for degradation of tryptophan

(i) Tryptophan 2,3-dioxygenase or indolamine 2,3-dioxygenase (ii) Kynurenine 3-hydroxylase (iii) Kynureninase (iv) Kynureninase (v) Kynurenine aminotransferase (vi) Kynurenine aminotransferase (vii) 3-Hydroxyanthranilic acid oxidase (viii) Aminomuconate semialdehyde decarboxylase
6.2 CLINICAL CASE HISTORY

Patient DN is a 13 year old female and only child of unrelated Caucasian parents. There is a family history of epilepsy and autistic spectrum disorder on the maternal side (Figure 56). She was delivered at full term following an uneventful pregnancy; her neonatal period and early development were unremarkable.

Figure 56. Family pedigree of patient DN

6.2.1 Seizures

The first onset of generalised tonic clonic (GTC) seizures was at 17 months of age associated with fever. She subsequently developed febrile and afebrile GTC and absence seizures which were difficult to control with conventional anticonvulsant medications. Eventually by late childhood her seizures were controlled on sodium valproate and she had only 3-4 breakthrough absence seizures per month. Several electroencephalogram (EEG) recordings throughout this period were normal. A brain MRI initially undertaken at 2 years of age showed a relatively large cisterna magna but was otherwise normal. Repeat imaging at 10 and 12 years of age demonstrated a stable tectal plate glioma which was presumed to be an incidental finding.
6.2.2 Episodic ataxia

Patient DN had an acute onset of ataxia at age 5 years in association with fever, upper respiratory tract symptoms and elevated inflammatory markers. She suffered subsequent exacerbations of ataxia with fever, illness and fatigue, often accompanied by withdrawn and obsessive behaviour. This picture was on the background of a chronic intention tremor and poor co-ordination. These symptoms were particularly severe in the morning and predominantly affected the upper limbs. They were not associated with anticonvulsant toxicity as evidenced by blood monitoring.

6.2.3 Sleep disorder

Severe disruption of circadian rhythm began in late childhood with the inability to sleep more than 3 hours at night and significant daytime somnolence. Unfortunately this was not responsive to behavioural intervention or melatonin. Telemetry and sleep latency tests undertaken at 13 years of age showed multiple arousals and no evidence of narcolepsy.

6.2.3 Asperger’s Syndrome

Asperger’s was diagnosed at 9.7 years of age by 3Di assessment which showed impairment in all 3 areas of the autistic triad; (i) reciprocal social interaction skills, (ii) use of language and other social skills and (iii) repetitive/stereotyped behaviour and routines in the presence of normal early language development and above average intelligence (WISC III verbal IQ 131).

6.2.4 Response to vitamin B₆ treatment

Following the finding of a significantly reduced PLP level in cerebrospinal fluid (Table 41, Section 6.4) oral pyridoxine (100 mg twice daily; approximately 5 mg/kg/day) was commenced at 11 years. This was subsequently changed to PLP (30 mg/kg/day) due to the development of symptoms of peripheral neuropathy. Nerve conduction studies at this time were normal. On both forms of vitamin B₆ the patient showed cessation of absence seizures, tremor and ataxia and an improvement in her social functioning and sleep pattern.
6.3 METHODS

6.3.1 Biochemical analysis

Whole blood serotonin was analysed by HPLC in the Neurometabolic Diagnostic Laboratory, National Hospital for Neurology and Neurosurgery, Queen Square, London as described in Section 2.2.4 (289). Each sample was frozen at the bedside and stored at -80°C for no more than 4 months prior to collection.

All B₆ vitamers were measured in plasma by the author using HPLC linked to tandem mass spectrometry as described in Section 2.2.2. Each sample was spun within 1 hour of collection and plasma frozen at -80°C for a maximum of 4 months prior to analysis.

Plasma amino acids were measured on the day of sampling in the chemical pathology laboratories, Great Ormond Street Hospital, London by HPLC and serum albumin by a bromocresol green (BCG) dye binding method.

Neurotransmitter amine metabolites, pterins and PLP were analysed as described in Section 2.2.5 (293). CSF PLP concentration was analysed in the second 0.5ml of CSF.

Other biochemical and haematological parameters reported in Tables 40 and 41 were analysed in the routine diagnostic laboratories of Great Ormond Street Hospital, London.

6.3.2 Molecular genetic analysis; sequencing of the tryptophan 2, 3-dioxygenase (TDO2) gene (ENSG00000151790)

DNA was extracted from whole blood using a Puregene Genomic DNA Purification Kit as described in Section 2.3.1.

Polymerase chain reaction (PCR) primers were designed using the Primer3 design website (http://frodo.wi.mit.edu/); the coding exons and exon/intron boundaries, 5’ and 3’ untranslated regions and 850 bp of the upstream promoter region of the TDO2 gene were amplified using the intronic primers detailed in Table 39. Transcript ENST00000536354 of TDO2-001 was used to generate primers.
A typical PCR reaction is described in Section 2.3.2; details of annealing temperatures and MgCl$_2$ concentrations used for these primers are shown in Table 39. PCR products were cleaned and sequenced by Sanger sequencing as described in Section 2.3.3. Any nucleotide changes that were found were compared to the Ensembl database (www.ensembl.org).
Table 39. Primers and PCR conditions required for the amplification of human tryptophan 2, 3-dioxygenase (TD02)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Predicted product size (bp)</th>
<th>MgCl₂ conc. (mmol/L)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream promoter region*</td>
<td>S:5’ CTGCCTCAGCTCCCTCAGTA 3’ A/S:5’ CCCAAGAAAACATTTTGGCATA 3’</td>
<td>484</td>
<td>1.0</td>
</tr>
<tr>
<td>Upstream promoter region*</td>
<td>S:5’ GAAGTGAGGCTTTGAGCATGT 3’ A/S:5’ CATTTCATTTGGTACAGAACTGA 3’</td>
<td>370</td>
<td>1.5</td>
</tr>
<tr>
<td>Upstream promoter region*</td>
<td>S:5’ GATCAGCTCAGCTTGAATCTAATGAAATG 3’ A/S:5’ CCCAAGAAAACATTTTGGCATA 3’</td>
<td>300</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 1 including 5’ untranslated region</td>
<td>S:5’ TTTGCAATGAGAATTTTAATCACC 3’ A/S:5’ AACAAAAATTTGAACAACTGAAGTG 3’</td>
<td>361</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 2</td>
<td>S:5’ GATGCAGGTAAGCAGGCTA 3’ A/S:5’ GGTGACAGTTTTCTAATGCTCCT 3’</td>
<td>246</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 3</td>
<td>S:5’ TGATAGATGTTCCTCAGGGTTT 3’ A/S:5’ CCCCTTTGAGTTAAGAACAAA 3’</td>
<td>495</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 4</td>
<td>S:5’ TCTGGCACAATTTGAGGACTA 3’ A/S:5’ TGACTGAAACTCCATCTACATCAT 3’</td>
<td>373</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 5</td>
<td>S:5’ GAAACTTTGGTTTGAATTTTGAATCAGTGG 3’ A/S:5’ TCAATGCTGCTAAGTTGCTAATATTCC 3’</td>
<td>691</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 6</td>
<td>S:5’ TGTCAGGTCTCTTTCTCTCCTCCC 3’ A/S:5’ TCTCAGCAGGTTCTGACA 3’</td>
<td>596</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 7</td>
<td>S:5’ TGTTTCTGGAAACCATGGAAGA 3’ A/S:5’ TCAATCATGTCACATAGTCCAGAAGA 3’</td>
<td>366</td>
<td>1.0</td>
</tr>
<tr>
<td>Exon 8</td>
<td>S:5’ GATAGAAGACCTGAGTATGTTGGA 3’ A/S:5’ AATAACTGTCAGCAGGGAACACC 3’</td>
<td>499</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 9</td>
<td>S:5’ AAAACATTTGGTTTTCTCTTATAACATGC 3’ A/S:5’ GGGGGCATAAAACATTCTATT 3’</td>
<td>486</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 10</td>
<td>S:5’ ATAGCCAAGCTGGCCACAG 3’ A/S:5’ TTCATGAGCCACCCACAAAGTA 3’</td>
<td>450</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 11</td>
<td>S:5’ GCTGTCATATGAGGCTTTCC 3’ A/S:5’ CCAGAAAATGCTGACACTGGA 3’</td>
<td>398</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon 12 including 3’ untranslated region</td>
<td>S:5’ CCTTGAGATGCTCCCTCAGA 3’ A/S:5’ TCTTGGGTCTTAAAGATCCTAAACA 3’</td>
<td>956</td>
<td>1.5</td>
</tr>
</tbody>
</table>

[KEY: S - sense primer; A/S - antisense primer; PCR, polymerase chain reaction; *overlapping primer pairs]
6.4 RESULTS

Tables 40 and 41 show the biochemical and haematological investigations in patient DN. She had persistently low plasma tryptophan in the presence of normal serum albumin (Table 40) and no evidence of increased urinary tryptophan excretion. The rest of her plasma amino acid profile was within the normal reference range with the exception of a mildly elevated glycine which is likely secondary to valproate treatment. Whole blood serotonin measured on two occasions fell below the lower reference limit (Table 40).

The concentration of pyridoxal phosphate (PLP) in cerebrospinal fluid (Table 41) was reduced below the age related reference range as described in Chapter 3. The concentration of PLP in plasma (Table 40) was in the low normal range, as were the concentrations of the other B₆ vitamers. The alterations in the plasma B₆ profile while on supplementation reflect those seen in the reference control population on supplementation (see Chapter 4).
Table 40. Sequential investigations in patient DN showing variation with change in medications

<table>
<thead>
<tr>
<th>Medication</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12y 0m</td>
</tr>
<tr>
<td>Sodium Valproate</td>
<td>Sodium Valproate, Pyridoxine (100mg twice daily; 5 mg/kg/day)</td>
</tr>
<tr>
<td>Plasma albumin [37 – 50] (g/L)</td>
<td>40</td>
</tr>
<tr>
<td>Plasma tryptophan [30-80] (μmol/L)</td>
<td>17</td>
</tr>
<tr>
<td>Whole blood serotonin [600 – 1600] (nmol/L)</td>
<td>539</td>
</tr>
<tr>
<td>Plasma B6 vitamers (nmol/L)</td>
<td></td>
</tr>
<tr>
<td>PLP (46.4 - 350.1)</td>
<td>54.5</td>
</tr>
<tr>
<td>PL (5.8 – 30.1)</td>
<td>6.4</td>
</tr>
<tr>
<td>PA (17.6 – 123.2)</td>
<td>38.6</td>
</tr>
<tr>
<td>PM (nd)</td>
<td>nd</td>
</tr>
<tr>
<td>PMP (nd – 9.6)</td>
<td>5.1</td>
</tr>
<tr>
<td>PN (nd – 0.6)</td>
<td>0.5</td>
</tr>
<tr>
<td>PNP (nd)</td>
<td>nd</td>
</tr>
</tbody>
</table>

KEY: Values outside the reference range shown in bold typeface; reference ranges shown in italics; nd, not detectable; y – years; m - months
Table 41. Additional laboratory investigations in patient DN

<table>
<thead>
<tr>
<th>Cerebrospinal Fluid</th>
<th>Pyridoxal phosphate 6 (10 – 37) nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose, lactate, SHIAA, HVA, 5-MTHF, pterins and amino acids (including tryptophan) - normal</td>
</tr>
<tr>
<td>Urine</td>
<td>Amino acids – normal excretion, normal sulphite</td>
</tr>
<tr>
<td></td>
<td>Organic acid profile – normal profile</td>
</tr>
<tr>
<td></td>
<td>α-Aminoadipic semialdehyde – normal excretion</td>
</tr>
<tr>
<td>Genetics</td>
<td>Pyridox(am)ine 5’ phosphate oxidase (PNPO) – no mutations</td>
</tr>
<tr>
<td></td>
<td>Spinocerebellar ataxia type 6 (SCA6) – no expansion</td>
</tr>
<tr>
<td></td>
<td>Episodic ataxia type 2 – no mutations</td>
</tr>
<tr>
<td>Other normal</td>
<td>Liver function tests</td>
</tr>
<tr>
<td>investigations</td>
<td>Alkaline phosphatase, albumin</td>
</tr>
<tr>
<td></td>
<td>C-reactive protein</td>
</tr>
<tr>
<td></td>
<td>Thyroid function tests</td>
</tr>
<tr>
<td></td>
<td>Full blood count</td>
</tr>
<tr>
<td></td>
<td>Red cell folate</td>
</tr>
<tr>
<td></td>
<td>Serum vitamin B₁₂ and vitamin D</td>
</tr>
<tr>
<td></td>
<td>Serum copper</td>
</tr>
<tr>
<td></td>
<td>Anti-streptolysin O titre</td>
</tr>
<tr>
<td></td>
<td>Anti-basal ganglia antibodies-low titres</td>
</tr>
<tr>
<td></td>
<td>Parvovirus and varicella serology</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex CFT &lt;10</td>
</tr>
<tr>
<td></td>
<td>Anti-nuclear antibodies</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td></td>
<td>Anti-thyroid peroxidase antibody</td>
</tr>
<tr>
<td></td>
<td>IgA endomysial antibody</td>
</tr>
</tbody>
</table>

Reference range in italics

Sequencing of the coding and upstream promoter regions of the TDO2 gene revealed ten sequence variations which are shown in Table 42. Eight in the non-coding intronic region have been previously reported on the Ensembl database ([www.ensembl.org](http://www.ensembl.org)). A splice site prediction tool ([www.fruitfly.org](http://www.fruitfly.org)) suggested that rs72681567 which is the only SNP lying close (16 bp) to an intron-exon boundary does not create a new cryptic splice site.

Of the two unreported changes one lies deep within an intronic region at base position 156838470 and is unlikely to affect protein expression (c.897G -142A>T). The second lies within Exon 8 (Table 42) and results in an amino acid change from a serine to a cysteine in a well conserved area of the protein (c.740C>G p.Ser247Cys) (Figure 57). Its potential significance is discussed in later sections.
Table 42. Sequence variations and single nucleotide polymorphisms (SNP) in the TDO2 gene of patient DN

<table>
<thead>
<tr>
<th>Sequence variant with SNP identifier where known</th>
<th>Position in gene</th>
<th>Variation</th>
<th>Sequence variation in patient DN</th>
<th>Population frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3775086</td>
<td>Intronic</td>
<td>T/A</td>
<td>T/A heterozygote</td>
<td>96% T; 4% A</td>
</tr>
<tr>
<td>rs3755909</td>
<td>Intronic</td>
<td>C/T</td>
<td>C/T heterozygote</td>
<td>95% C; 5% T</td>
</tr>
<tr>
<td>rs2137778</td>
<td>Intronic</td>
<td>C/G</td>
<td>G/G homozygous</td>
<td>98% G; 2% C</td>
</tr>
<tr>
<td>rs72681567</td>
<td>Intronic</td>
<td>A/G</td>
<td>A/G heterozygote</td>
<td>93% G; A 7%**</td>
</tr>
<tr>
<td>rs34133854</td>
<td>Intronic</td>
<td>T/C</td>
<td>T/C heterozygote</td>
<td>95% T; 5% C</td>
</tr>
<tr>
<td>rs10517626</td>
<td>Intronic</td>
<td>G/T</td>
<td>G/T heterozygote</td>
<td>94% G; 6% T</td>
</tr>
<tr>
<td>rs12502494</td>
<td>Intronic</td>
<td>C/T</td>
<td>C/T heterozygote</td>
<td>92% T; 8% C</td>
</tr>
<tr>
<td>Unreported variant, base 156835488</td>
<td>Exonic</td>
<td>C/G</td>
<td>C/G heterozygote</td>
<td>unknown</td>
</tr>
<tr>
<td>Unreported variant, base 156838405</td>
<td>Intronic</td>
<td>T/A</td>
<td>T/A heterozygote</td>
<td>unknown</td>
</tr>
<tr>
<td>rs2292537</td>
<td>Intronic</td>
<td>C/T</td>
<td>T/T homozygote</td>
<td>90% T; 10% C</td>
</tr>
</tbody>
</table>

*from 1000 Genomes project; reported for European ancestry (CEU) when information available

**Japanese population
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>EFIRIQAKEESEEKEEQVAEFQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Gibbon</td>
<td>EFIRIQAKEESEEKEEQVAEFQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>EFIRIQAKEESEEKEEQVAEFQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Gorilla</td>
<td>EFIRIQAKEESEEKEEQVAEFQKQKEVLLSLF 271</td>
</tr>
<tr>
<td>Macaque</td>
<td>EFISIQAKEESEEKEEQVAEFQKQKEVLLSLF 264</td>
</tr>
<tr>
<td>Guinea</td>
<td>EFIKIQAKEESEEKEEQLQKRREVLLSLF 267</td>
</tr>
<tr>
<td>Rabbit</td>
<td>EFIKIQAMEESEEKEEQVAEFQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Cow</td>
<td>EFTKIQAKEESEEKEEQLQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Mouse</td>
<td>EFLRIKAKTDSEEKEEQMAEFQKQKEVLLCLF 268</td>
</tr>
<tr>
<td>Rat</td>
<td>EFLKIQAKKDSEEKEEQMAEFQKQKEVLLCLF 268</td>
</tr>
<tr>
<td>Panda</td>
<td>EFIKIQAKEESEEKEEQLQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Dog</td>
<td>EFIKIQAKEESEEKEEQLQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Dolphin</td>
<td>EFIKIQAKEESEEKEEQLQKQKEVLLSLF 267</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>ELIRIQAKEESEEKEEQLQKQKEVLLSLF 266</td>
</tr>
<tr>
<td>Chicken</td>
<td>EFAIQCEQESEDLLSEFQKQDTLLSLF 268</td>
</tr>
<tr>
<td>Anole Lizard</td>
<td>EFAMVQAKDSELKDQLAELQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Bush Baby</td>
<td>DFIRIQAREESEEKEEQLQKQKEVLLSLF 268</td>
</tr>
<tr>
<td>Cod</td>
<td>EKEKIEGMAEDELKQKQKEVFTSLF 269</td>
</tr>
</tbody>
</table>

Figure 57. Sequence alignment of tryptophan 2, 3 dioxygenase protein across species showing conservation of human serine 247
6.5 DISCUSSION

Patient DN presents with a syndrome of tryptophan, serotonin and pyridoxal phosphate deficiency in association with epilepsy, ataxia and Asperger’s syndrome which may represent an abnormality at the level of the enzyme tryptophan 2, 3-dioxygenase. The results of her biochemical and molecular genetic investigations are discussed below in the context of tryptophan metabolism and in the light of previously reported clinical cases which demonstrate similar features.

6.5.1 Tryptophan metabolism

The essential amino acid Trp is present in relatively low concentrations in plasma compared to other amino acids and forms only a small proportion of tissue proteins (421). Dietary deficiency of Trp is extremely rare in the developed world as it is present in most protein containing food groups such as red meat, fish, egg and peanuts and is particularly abundant in chocolate. The clinical presentation seen in dietary tryptophan deficiency relates specifically to the resulting nicotinamide depletion and is characterised by the three D’s; ‘dermatitis, diarrhoea and dementia’. This deficiency state named ‘pellagra’ is classically described in areas where maize is the staple diet, as nicotinamide (niacin) is not biologically available in maize unless it is specifically prepared with an alkali treatment.

Trp is an interesting amino acid because it is involved in diverse metabolic pathways and thus impacts upon various physiological processes. Given its low plasma concentration, yet the diversity and importance of its actions, Trp homeostasis is vulnerable to disruption and the contribution of the different pathways of metabolism will vary according to physiological and pathological state (421).

Trp circulates bound to albumin and only 10 – 20 % is unbound or ‘free’ Trp (422). Several factors can affect Trp binding, displacing it from albumin; these include drugs such as benzodiazepines and high concentrations of non-esterified fatty acids (NEFA) which may follow activation of the sympathetic nervous system, for example (173). What effect Trp binding has on the biological availability of the amino acid remains controversial (423), however albumin bound Trp cannot cross the blood brain barrier.

Many observations demonstrate that peripheral Trp metabolism can alter serotonin synthesis in the brain (424), thus there is great interest in the mechanisms available for Trp uptake into
the central nervous system and the factors that may affect it. Trp crosses plasma membranes, including those at the blood brain barrier, by a neutral amino acid transporter (LAT1) which is activated by the heavy chain of 4F2 antigen (409;410). Studies have shown that this sodium independent transport system can transport large amino acids with a branched or aromatic side chain and that it shows high stereoselectivity for Tyr, Trp, Val and Histidine. The various amino acid substrates compete for transport and experiments suggest that the transporter acts as an amino acid exchanger at the blood brain barrier allowing bi-directional transport of amino acids.

6.5.2 Metabolic fates of tryptophan

Aside from its role in protein formation, Trp has two non-protein metabolic fates; <5% is metabolised to serotonin via the methoxyindole pathway and the remaining majority enters the kynurenine pathway (418). The metabolic pathway for serotonin synthesis is discussed in detail in Chapter 1, Section 1.2. In addition to its function as a neurotransmitter, in the pineal gland serotonin serves as the precursor for melatonin, a neuro-hormone responsible for sleep onset during darkness and hence regulation of circadian rhythm.

Interest in metabolites lying on the kynurenine pathway has increased over recent years as they have been implicated in many and varied aspects of pathophysiology. The main pathway shown in Figure 55 illustrates that the principle branch of tryptophan oxidation can either generate fuel for energy production in the form of acetyl CoA or lead to the net new synthesis of nicotinamide nucleotides, NAD and NADP (425). This pathway also has side branches that form kynurenic acid and xanthenuric acid. Two of the enzymes (kynurenine aminotransferase and kynureninase) are pyridoxal phosphate dependent and, prior to advanced analytical methods discussed in Chapter 3, detection of xanthenuric acid in urine following a tryptophan load was widely used as an indicator of B₆ deficiency. It is also interesting to note that quinolinic acid and picolinic acid, which have important effects in the brain, are formed non-enzymatically and thus are only produced when there is a large amount of flux through the pathway (425).

The effect of three kynurenine pathway metabolites within the central nervous system are of particular interest; namely quinolinic acid, kynurenic acid and 3-hydroxykynurenic. Quinolinic acid has been shown to selectively activate NMDA receptors producing excitation and axon-sparing lesions in the brain (426;427). It is pro-convulsant in animal models.
and demonstrates several mechanisms of neurotoxicity in addition to NMDA receptor activation including production of mitochondrial dysfunction and free radical generation. Given the important role of NMDA receptors in guiding axonal connections and synaptogenesis in the developing brain, a change in the physiological concentration of this metabolite during development may feasibly cause disease. 3-Hydroxykynurenine also shows toxicity towards neurones but probably by differing mechanisms (430).

3-Hydroxykynurenine appears to initiate apoptosis as a result of conversion to quinonimines (431) and possibly also following its conversion to 3-hydroxyanthranilic acid which is known to be destructive (418). Kynurenic acid has apparently opposing effects within the CNS as this metabolite can block glutamate receptors and is an antagonist at both the glutamate binding site and the allosteric glycine site of the NMDA receptor (432;433).

Although all enzymes of the kynurenine pathway are expressed within the CNS, studies in rats have shown that peripheral production of some kynurenine metabolites also contributes significantly to the cerebral pool (434). Kynurenine is readily taken up by the large neutral amino acid transporter, competing with other amino acids to enter the brain as described above. In addition both 3-hydroxykynurenine and anthranilic acid can pass the blood brain barrier, possibly by the same mechanism, whereupon they can undergo further metabolism to their downstream potentially neurotoxic products. In contrast, the effect of peripheral quinolinic acid, kynurenic acid and 3-hydroxyanthranilic is very limited due to their restricted blood brain barrier transport (434).

It is clear that any alteration in the concentration of these metabolites within the circulation or CNS could play a role in a variety of neurological diseases. An increased serum kynurenine:Trp ratio has recently been reported as evidence for ‘activation’ of the kynurenine pathway in adolescent anhedonia (inability to experience pleasure observed in many mental health disorders) (435) and idiopathic generalised epilepsy (436). Increased kynurenic acid has been described in cerebrospinal fluid of male schizophrenic patients (437), the vast majority of whom were drug naïve, and, more recently, evidence of increased expression of tryptophan dioxygenase (mRNA) has been demonstrated in post-mortem brain of patients with schizophrenia and bipolar disorder (438).
6.5.3 Tryptophan 2, 3 dioxygenase enzyme

Man has two distinct oxygenase enzymes involved in the degradation of Trp; tryptophan 2,3-dioxygenase (TDO) which is primarily expressed in liver (and astrocytes) (438) and indolamine 2,3-dioxygenase (IDO) which is active in non-hepatic tissues. Plasma Trp concentrations are influenced by TDO activity (439) as it is the rate-limiting enzyme for Trp degradation via the kynurenine pathway (173;440;441). TDO is a homotetrameric enzyme containing two haem units per tetramer. It is iron and copper dependent and shows remarkable evolutionary conservation. TDO acts by inserting molecular oxygen into the pyrrole moiety of Trp yielding formylkynurenine as the reaction product (442) and, despite catalysing the same biochemical reaction, it shows surprisingly little (<10%) sequence similarity with IDO (440). TDO is highly specific for L-tryptophan as a substrate and extensive studies of TDO in Xanthomonas campestris have now demonstrated the mechanisms of substrate recognition and catalysis of this enzyme (440;443). How substrate and \( \text{O}_2 \) binding is controlled however, is still largely unknown (444).

The activity of TDO may be increased by Trp and \( \alpha \)-methyl tryptophan via actions at an allosteric site which lies away from the enzyme’s active site (442). Trp appears to increase enzyme activity by stabilising the enzyme complex and preventing degradation. In addition, the administration of corticosteroids has been shown to result in a 10- to 15-fold increase in TDO mRNA (445). Increased activity of TDO (due to increased protein production) associated with a proportional increase in kynurenine excretion was demonstrated by enzyme assay in the liver biopsies of a group of hospitalised patients (446). Activation of the pathway was considered likely secondary to the ‘stress response’ in these patients who had a variety of different diagnoses. Conversely, TDO is competitively inhibited by 5-HTP, melatonin and the final end-product of the pathway nicotinamind adenine dinucleotide phosphate and its analogues.

6.5.4 Tryptophan 2, 3 dioxygenase gene (\( TDO2 \))

Given that several factors such as haem and copper are required for optimum enzyme activity and that others such as corticosteroids can upregulate enzyme activity, there are multiple regions of the \( TDO2 \) gene where mutations could result in altered enzyme activity. Sequencing of this gene in patient DN therefore included the upstream promoter and regulatory region in addition to the exonic protein coding regions.
The TDO2 gene in man is localised on chromosome 4q31 and is composed of twelve exons (447). In both the rat and human sequence there is a large regulatory component and in rat it contains two glucocorticoid response elements (GRE) and a globin promoter region. In man at least one GRE is present and there is insertion of an additional region of non-homologous sequence containing a putative alternative second GRE element (447). Possible haem binding histidine residues have been identified in both the human and rat protein sequence.

Comings et al. (448) have demonstrated a significant association with some TDO2 polymorphisms in various neuropsychiatric conditions (Tourette syndrome, attention deficit hyperactivity disorder and drug dependence) suggesting a role for TDO in these likely polygenic disorders (448). Of particular interest to the patient reported here, is the work of Nabi et al. (449), who investigated five single nucleotide polymorphisms in the TDO2 gene for association with autism. Results of the study suggest the presence of a susceptibility mutation in the TDO2 gene in the 196 multiplex families tested and revealed linkage disequilibrium of a promoter variant (rs3755910, not present in patient DN) with autism. Clinical data for the cohort examined is not detailed, but the group included patients with a diagnosis of autism, Asperger syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS). Information about co-morbid features is not provided. The hypothesis of reduced TDO activity is proposed by the group, citing it as a possible mechanism for the well documented hyperserotonaemia in autism. For Patient DN however, increased activity of the enzyme could account for the biochemical findings as discussed below.

6.5.5 Clinical cases of abnormal tryptophan and kynurenine metabolism

Trp deficiency is rarely encountered in clinical practice although it has been described in a group of children with Tourette syndrome, of uncertain significance (450). The only inborn error of metabolism that causes low plasma Trp and is well understood is Hartnup disorder. This is an autosomal recessive disease resulting in deficiency of a neutral amino acid transporter at the epithelial surface of intestinal and renal tubular cells. This defect results in a striking neutral hyperaminoaciduria (alanine, serine, threonine, valine, leucine, isoleucine, phenylalanine, tyrosine, histidine, citrulline, asparagine, glutamine and tryptophan) and corresponding decreased plasma amino acid concentrations (417). The clinical features of pellagra-like dermatitis and neurological deterioration are also characteristic and are attributed to nicotinamide deficiency secondary to tryptophan depletion. Patients with
Hartnup disorder may be successfully treated with oral nicotinamide and adequate protein supplementation.

Perhaps surprisingly, there are few described inborn errors lying on the tryptophan or kynurenine metabolic pathways. Those reported seem to have an inconsistent disease phenotype; none have been proven by enzymology and only one case has diagnostic confirmation by genetic mutational analysis. They are summarised in Table 43.

Based on biochemical findings of elevated urinary kynurenine, 3-hydroxykynurenine and xanthenuric acid and reduced anthranilic acid, Komrower et al. (451) described a patient with a likely disorder of tryptophan metabolism. Her symptoms of diarrhoea, stomatitis, poor growth and headaches responded to nicotinamide but not pyridoxine supplementation, suggesting a diagnosis of kynureninase deficiency. The resolution of her early childhood haemolytic anaemia is not so easily attributable to nicotinamide deficiency however, which due to technical difficulties, could not be demonstrated on biochemical analysis of urine. The authors postulate that accumulation or deficiency of one of the other kynurenine metabolites may explain this feature however it has not subsequently been reported in any other patient with a possible disorder of tryptophan oxidation. More recently a child with a different presentation of incidental asymptomatic xanthenuric aciduria was described and shown to have homozygous mutations in the kynureninase gene (452) perhaps suggesting dual pathologies in the first case.

Clayton et al. (453) reported biochemical evidence of kynurenine hydroxylase deficiency in a 9 year old girl who had a classical clinical picture of pellagra; dermatitis, diarrhoea and dementia. Her symptoms improved dramatically with nicotinamide (but not with nicotinic acid) and showed no response to the cofactor for kynurenine hydroxylase, FAD (in the form of riboflavin).

Two families with mental retardation, volatile affect and speech abnormalities have been described in whom hypertryptophanaemia and elevated excretion of indoleic acids may represent a failure to convert tryptophan to kynurenine (454;455). A diagnosis by mutational analysis was not pursued in either family. Wong et al. (456) also report biochemical evidence of reduced activity of tryptophan oxygenase in a patient with a different clinical phenotype of pellagra-like rash and cerebellar ataxia.
Salih et al. (457) report a severe familial condition which is lethal in early childhood and may result from abnormally high activity of picolinate carboxylase. This condition is characterised by a pellagra-like skin condition, neurological impairment including cerebellar signs, severe sleep disturbance and the development of cataracts.
Table 43. Summary of published clinical cases for possible inherited defects of tryptophan metabolism

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>1 child</td>
<td>1 child</td>
<td>1 child</td>
<td>1 child</td>
<td>2 siblings</td>
<td>10 cases (single family)</td>
<td>1 child</td>
<td>1 child (asymptomatic)</td>
</tr>
<tr>
<td>Photosensitive dermatitis</td>
<td>●</td>
<td>No</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>No</td>
</tr>
<tr>
<td>Cerebellar ataxia</td>
<td>●</td>
<td>No</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Short stature</td>
<td>●</td>
<td>●</td>
<td>Failure to thrive</td>
<td>●</td>
<td>●</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>●</td>
<td>Low IQ</td>
<td>● Low IQ</td>
<td>Not reported</td>
<td>● Low IQ</td>
<td>●</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Neuropsychological dysfunction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Depression, Family history schizophrenia</td>
<td>Insomnia</td>
<td>Anxiety, depression, paranoia</td>
</tr>
<tr>
<td>Colitis, diarrhoea</td>
<td>●</td>
<td>No</td>
<td>●</td>
<td>No</td>
<td>●</td>
<td>No</td>
<td>●</td>
<td>No</td>
</tr>
<tr>
<td>Haemolytic anaemia</td>
<td>-</td>
<td>●</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Response to nicotinamide</td>
<td>Not reported</td>
<td>Growth and headaches improved</td>
<td>Rash improved</td>
<td>Rash and ataxia improved</td>
<td>Not reported</td>
<td>Exacerbation of rash with nicotinic acid</td>
<td>Rapid improvement all symptoms</td>
<td>-</td>
</tr>
<tr>
<td>Plasma tryptophan</td>
<td>Normal</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
<td>Reduced (normal 5-HTP)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Whole blood serotonin</td>
<td>-</td>
<td>-</td>
<td>Normal</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>Urinary amino serotonin excretion</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Urinary Kyn, 3HK, XA</td>
<td>Reduced Kyn excretion</td>
<td>Increased excretion Kyn, 3HK and XA</td>
<td>No excretion anthranilic acid</td>
<td>Reduced excretion Kyn post Trp load</td>
<td>KA and XA excretion reduced post Trp load</td>
<td>Reduced N methyl nicotinamide and 3-hydroxyanthranillic acid</td>
<td>Elevated kynurenine; reduced KA, XA. No quinolinic acid present</td>
<td>Increased excretion Kyn and KA; reduced excretion 3HK</td>
</tr>
<tr>
<td>Proposed defect</td>
<td>Defect in Trp conversion to Kyn</td>
<td>Kynureninase deficiency</td>
<td>Reduced activity tryptophan oxygenase</td>
<td>Block in tryptophan degradation</td>
<td>-</td>
<td>Increased activity picolinate carboxylase</td>
<td>Kynurenine hydroxylase deficiency</td>
<td>Homozygous mutation in kynureninase gene</td>
</tr>
</tbody>
</table>

- not reported or not measured; ● clinical feature present; kyn – kynurenine; 3HK – 3-hydroxykynurenine; XA – xanthenuric acid
6.5.6 Patient DN

It is possible that patient DN described in this chapter has an inborn error of tryptophan metabolism given the persistent abnormal finding of reduced plasma tryptophan. Tryptophan binding to albumin may affect its biological actions as discussed above, however the laboratory method used here measures total tryptophan and therefore excludes factors which alter binding as causative.

Patient DN does not fit the clinical or biochemical phenotype for Hartnup disorder, the only well described disorder involving tryptophan. Specifically in her case, the tryptophan deficiency is isolated, the urinary amino acid profile is normal and there are additional abnormalities evident in blood and cerebrospinal fluid (Tables 40 and 41). She has no skin abnormalities therefore it is unlikely that the low plasma tryptophan is causing nicotinamide deficiency i.e. there is not a deficiency in a final product of this pathway. She does however share some of the other clinical features described in presumed cases of inborn errors of tryptophan metabolism including ataxia, sleep disturbance and neuropsychiatric symptoms. In particular the cases described by Fenton et al. (458) have a seemingly identical picture of tremor and ataxia which is worse in the morning and exacerbated during times of fever and infection. There are no cases reported with the same biochemical findings, however it is useful to consider her clinical problems in light of the laboratory findings.

Plasma Trp concentrations are regulated by TDO which is known to be an inducible enzyme in some situations. Conceivably an increase in the activity of this enzyme, which is inappropriate to the physiological state, may lead to reduced plasma tryptophan and increased flux through the kynurenine pathway. The resulting increase in kynurenine metabolites, particularly kynurenine, 3-hydroxykynurenine and anthranilic acid may enter the central nervous system (CNS) with deleterious consequences such as seizure generation. In keeping with this, the whole blood serotonin concentration in this patient was below the lower limit of the laboratory reference range and is very different to our paediatric control range (Chapter 4), implying a diversion of tryptophan away from indolamine synthesis. It is surprising that measurement of CSF tryptophan and 5-HIAA provided no evidence of tryptophan or serotonin deficiency in the CNS, as some of patient DN’s problems, such as sleep disorder, could be readily explained by reduced serotonin and hence
melatonin synthesis in the brain. This may be because tryptophan entry into the CNS fluctuates and the plasma and CSF samples documented here were not drawn simultaneously. It should also be borne in mind that it is the catabolic product of serotonin (5-HIAA) that is measured in CSF rather serotonin itself. An important abnormality in the biochemical profile of the CSF sample obtained from DN was the low concentration of PLP. This and the response to B6 supplementation strongly suggest a defect in the synthesis of PLP. Two studies have shown that the kynurenine metabolites (3HK, 3-hydroxyanthranilic acid, xanthenuric acid, picolinic acid and quinolinic acid) inhibit pyridoxal kinase which is the enzyme responsible for catalysing the formation of PLP from PL (419;420). This mechanism may account for the severe central PLP deficiency observed and the improvement on vitamin B6 treatment. Absence of the skin condition pellagra caused by nicotinamide deficiency in this patient is in keeping with increased, rather than reduced flux through the kynurenine pathway and it is interesting to note that a recent study found evidence of increased urinary excretion of N-methyl nicotinamide and N-methyl nicotinic acid in a group of autistic children by NMR spectroscopy (405).

Theoretically, an inappropriate increase in the activity of TDO may arise by a number of different mechanisms. It may be due to an increase in one or more factors known to stimulate protein expression such as corticosterone release or it may be due to an abnormality at a genetic level; a ‘gain-of-function’ mutation. Given the numerous factors that can affect TDO enzyme activity there are multiple regions of the TDO gene where a mutation could result in increased activity, particularly in the regulatory and substrate binding regions.

Coding regions and upstream regulatory regions of the TDO2 gene were sequenced in patient DN. The nine single base changes lying in intronic regions are unlikely to affect expression of the protein.

A single heterozygous base change, not previously reported was found in exon 8 which results in non-synonymous coding, changing a serine to a cysteine (S247C). Serine 247 lies in a highly conserved region of the TDO2 protein (Figure 57) indicating that it is functionally important and although serine and cysteine differ by only one atom, they are chemically distinct as cysteine contains a sulphydryl group where serine has a hydroxyl group. Cysteine often forms disulphide bridges within a
protein and is important for the primary structure formation, whereas serine can form hydrogen bonds and forms the secondary structure of proteins.

6.6 Future Considerations

This amino acid change is potentially interesting in the context of the biochemistry results in patient DN and warrants further investigation. Initially the heterozygous base change should be confirmed in the patient and her parents (unfortunately parental DNA is not currently available). In addition a cohort of 100 ethnically matched controls should be screened to ensure it is not a common polymorphism that has yet to be reported. Following this, functional studies of the mutant protein could be undertaken.

Analysis of plasma and CSF kynurenine metabolites and their ratio to tryptophan in patient DN would further support the genetic investigations and could be measured by tandem mass spectrometry. It would also be interesting to look for evidence of kynurenine pathway disruption in other patients who share similar clinical features with Patient DN; although plasma Trp reduced below normal reference ranges has not been well described in autism or Asperger’s syndrome, the ratio of Trp to kynurenine rather than the absolute concentration may be altered with pathological consequences of kynurenine metabolites in the central nervous system.

If the kynurenine pathway is proven to be implicated in the case described here then potential options for treatment should be considered. Possible pharmacological agents include the non-steroidal anti-inflammatory agents (tolmetin and sulindac) which are commonly used for treatment of pain in arthritis. Both were shown to inhibit liver tryptophan 2, 3-dioxygenase and increase brain serotonin and melatonin levels in rats (459). Other work in animals has identified several different compounds which prevent seizure induction by the inhibition of kynureninase and kynurenine 3-hydroxylase. Their action diverts Trp away from quinolinic acid and towards kynurenic acid thus offering neuro-protection (460;461).
Chapter 7
Summary and Further Work
The complex metabolism and diverse functions of vitamin B₆ make it an interesting and challenging area of paediatric medical research. One important synthetic pathway in which it plays a role is in the production of serotonin via the PLP-dependent enzyme aromatic amino acid decarboxylase. Both vitamin B₆ and serotonin metabolism have been addressed in the research presented within this thesis with respect to two common and disabling neurological disorders of childhood; epilepsy and autism. They share in common a heterogeneous clinical presentation and diverse aetiology. Although an understanding of the genetic basis for some epilepsy syndromes has advanced in recent years, much is yet to be learned about both conditions. Ultimately it is hoped that research in these patient groups will impact upon children and their families by improving diagnosis and treatment options and thereby quality of life.

Summary of the important findings of this work:

- Revision of the normal range for pyridoxal 5’-phosphate in cerebrospinal fluid
- Development and validation of an LC-MS/MS method for measurement of plasma B₆ vitamers and demonstration of its use in patients with B₆ responsive epilepsy
- Whole blood serotonin is not elevated in a group of high functioning autistic children and young adults (mean age 12 years 1 month) but aromatic L-amino acid decarboxylase activity in plasma is reduced. A hypothesis that this observation may be related to elevated 5-hydroxytryptophan has been proposed

The work presented in Chapters 3 and 5 highlights the importance of accurate age dependent metabolite reference ranges for diagnosis. It is apparent that during a period of rapid growth and development in the first months of life vast alterations take place in the metabolic milieu, reflected in age-dependent metabolite concentrations. This has important implications for diagnosis and treatment. Extrapolating this further to consider the development of metabolic pathways in utero and how premature delivery may impact upon this is another, as yet unexplored area for research.

Considering the metabolic pathways of vitamin B₆ in particular, work in animal models suggests developmental changes of pyridoxamine 5’-phosphate oxidase
during fetal life (30). In addition we know that \( B_6 \) has important antenatal effects as illustrated by inborn errors of metabolism that lead to pyridoxal 5’-phosphate deficiency, for example structural neuroanatomical changes and intrauterine seizures seen in some patients with Antiquitin deficiency. Very little is known about the normal range for plasma \( B_6 \) vitamers in the term newborn infant and even less about how this may differ in premature infants. A recent abstract documenting the measurement of \( B_6 \) vitamers in CSF suggests that differences do exist (462) although the reasons for this are unclear.

In this paediatric group nutrition is also of crucial importance. Artificial feeds (formula milk and parenteral nutrition), not uncommonly used in preterm infants, contain large amounts of pyridoxine as their predominant \( B_6 \) vitamer source in contrast to human breast milk which contains pyridoxal and pyridoxal 5’-phosphate in lower concentrations. Previous work suggests that accumulation of some vitameric forms may occur in babies and infants on formula or parenteral nutrition and in the long term it will be important to determine if there are any detrimental effects of such deviations from normal physiology, considering in particular the documented direct toxicity of some \( B_6 \) forms in excess and the possible secondary effects of the elevated cofactor (PLP) concentrations on downstream pathways, leading to alteration of critical neurotransmitters for example.

Future work to establish reference ranges in the term and preterm newborn and to investigate the effect of feeding method has been given ethical approval and will form the basis of important work to further understanding in this area of \( B_6 \) metabolism. In order to make this project practically possible, work has begun to adapt the method to measure \( B_6 \) vitamers in plasma by LC-MS/MS for use on dried blood spots. This will have enormous advantages not only in the small amount of sample that is required, but also in ease of sampling and resulting stability of the analytes.

As presented and discussed in Chapters 3 & 4 many patients present to clinicians with \( B_6 \) responsive seizures that do not fall into any of the known diagnostic categories; measurement of plasma/whole blood and CSF \( B_6 \) vitamers is likely to be helpful in the identification and understanding of novel monogenic \( B_6 \) responsive seizure disorders in these groups. The advent of next generation sequencing has in
recent years made rapid sequencing of the whole exome or even genome, a realistic possibility. While advances have been made and new single gene disorders described using this technology, problems do exist, not least the difficulty in interpretation of the enormous amount of data that is generated with little normative data with which to compare it. The power of this technology may be best realised if used in combination with biochemistry techniques in groups of patients who share a clearly delineated clinical phenotype. Undiagnosed patients with B₆ responsive seizures are certainly amenable to this approach of whole genome sequencing coupled to measurement of B₆ vitamer profiles.

In Chapter 5 novel findings are presented in a group of autistic subjects which suggest disturbed tryptophan-serotonin metabolism. These results pose interesting questions for further research which would be best addressed in large patient cohorts, as well as in their siblings and parents. It would be appealing to use a different approach to such research in individuals with mental health/behavioural difficulties; to categorise by a defined set of behaviours (endophenotypes) rather than diagnosis as many broad areas are shared and one condition may evolve over the lifetime of an individual. At present there is an unmet need for investigation of such disorders from a biochemical perspective, which if combined with radiological and genetic techniques could provide a better understanding of neuropsychiatric disease processes. In attempting to understand the novel findings in children with high-functioning autism and Asperger’s syndrome documented in Chapter 5, hypotheses were formulated that suggest that further research into this group of patients could involve measurement of 5-hydroxytryptophan in plasma, further studies on the characteristics of AADC in plasma and investigation of molecular mechanisms controlling AADC expression in various cell types in the brain.

Disorders of tryptophan metabolism via the kynurenine pathway have historically been a neglected area of clinical paediatric research. This in part may reflect the fact that many of these metabolites are rarely, if ever, measured in clinical practice and gross abnormalities of tryptophan concentration are unlikely to be present. It is possible that disruption of the kynurenine:tryptophan ratio is of more importance. In view of the well described neurological effects of many of these compounds it is potentially very interesting to study their concentrations in targeted patient groups.
Development of a UPLC linked tandem mass spectrometry method is proposed to simultaneously measure 5-hydroxytryptohan, tryptophan and kynurenine in plasma and CSF.

Given the recent advances in laboratory technologies within both biochemistry and molecular biology, the research community is well placed to further understanding of complex diseases over the coming years. In particular, large, well designed, collaborative studies offer much promise to unravel one of the biggest remaining challenges of human disease, the neuropsychiatric disorders.
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