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METABOLIC INVESTIGATION OF CEREBROSPINAL FLUID IN CHILDHOOD

amino acids, purines and pyrimidines in brain disease

Peter Gerrits

METABOLIC INVESTIGATION

OF CEREBROSPINAL FLUID

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Abbreviations

ASA:	argininosuccinic aciduria
ASL:	argininosuccinate lyase
BBB:	blood-brain barrier
CAPD:	continuous ambulatory peritoneal dialysis
CNS:	central nervous system
CRF:	chronic renal failure
CSF:	cerebrospinal fluid
ECF:	extracellular fluid
GABA:	γ-aminobutyric acid
HPLC:	high-performance liquid chromatography
ICF:	intracellular fluid
LNAA:	large neutral amino acids
MBP:	myelin basic protein
NMR:	nuclear magnetic resonance
NSE:	neuron-specific enolase
PRPP:	phosphoribosylpyrophosphate

Contents

Chapter 1

INTRODUCTION: CSF PHYSIOLOGY AND MODES OF EXCHANGE BETWEEN CSF, BRAIN AND BLOOD

Chapter 1

Introduction:
CSF physiology and modes of exchange between CSF, brain and blood

1.1 CSF-dynamics

The cerebrospinal fluid (CSF) is appropiately viewed as the clinician's access to the brain and is capable of reflecting pathophysiological changes in brain function [1].
The rate of formation in adults is approximately 0.35 ml per minute, or 500 ml per day [2]. The turnover of CSF, based on an average volume of 150 ml [3], is therefore 14 % per hour. The choroid plexus is the principal source of CSF. Evidence exists for extrachoroidal CSF excretion; the failure to relieve hydrocephalus by choroid plexotomy provides indirect evidence for extrachoroidal fluid formation in humans. There are indications for a flow of fluid from blood to CSF through the brain [4], and Cserr has demonstrated a substantial flow of brain extra cellular fluid (ECF) into CSF [5].

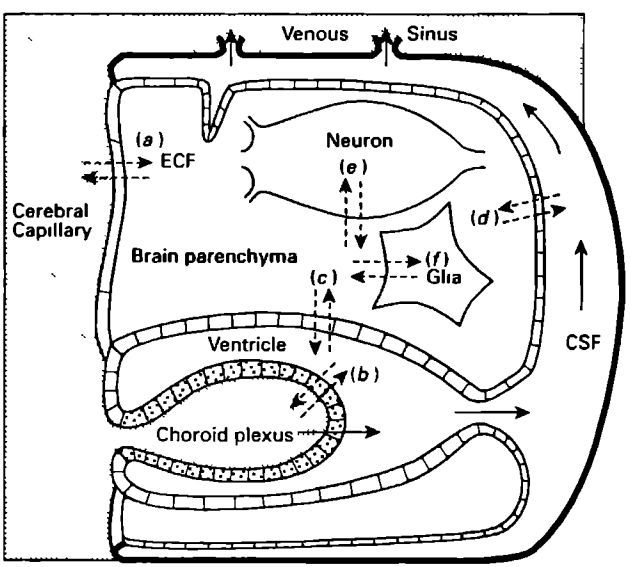

Figure 1.1 Diagram of fluid compartments of the blood-brain-CSF system. Continuous arrows represent proven directions of CSF flow. Interrupted arrows indicate where diffusion of water and solutes may occur between the different compartments: (a) across the blood-brain barrier, between brain capillaries and extracellular fluid; (b) across the epithelia of the chroroid plexuses; (c) across the ependyma; (d) across the piaglial membranes; (e) and (f) across the cell membranes of neurons and glial cells. Thick outline represents the arachnoid-dural enclosure of the system. Illustration from [10].

CSF is secreted by an incompletely understood two-step process [6]. First, fluid is filtered through the highly permeable core capillary of the choroidal frond into the extracellular space surrounding choroidal cells. Second sodium is actively transported across choroidal cells into CSF, and water follows obligatory down an osmotic gradient.

It is generally agreed that the major absorptive sites are the arachnoidal granulations which penetrate the major dural venous sinusus in the cranium [7, 8] (fig. 1.1). The mechanisms by which the CSF and its constituents are absorbed in bulk through the arachnoid villi are not clear.

Factors influencing CSF composition

The majority of compounds in CSF has a concentration which is much lower than that in blood (see following chapters). The concentration of amino acids, for example, is about 10-15 % of the plasma concentration, with the exception of glutamine (equal concentrations). Davson et al [9] suggested that, because of the very low concentrations in the CSF, the latter would act as a 'drain' or 'sink', continuously draining the brain tissue of the compounds acquired from the plasma (fig. 1.2).

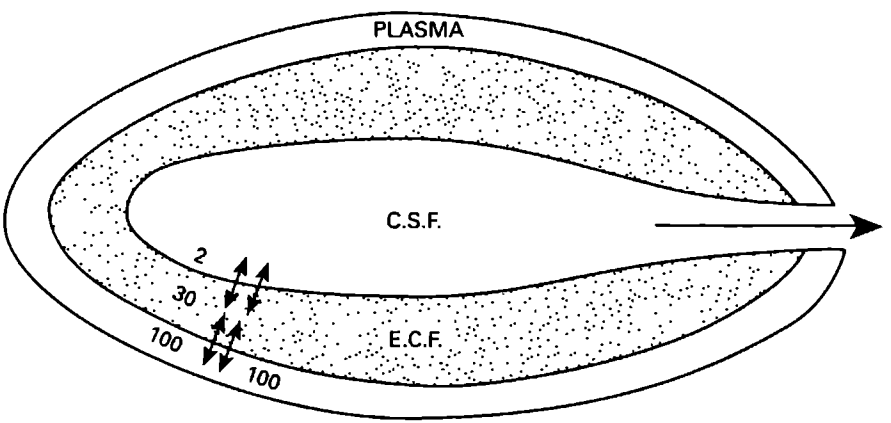

Figure 1.2 *Illustrating the 'sink-action' of the cerebrospinal fluid whereby the fluid imposes a low concentration on an extracellular tag in the brain. Thus if plasma concentration is 100, the cerebrospinal fluid concentration may be only 2 and the concentration in the extracellular fluid may be only 30 [10].*

This, so called, sink-action explains why, in intravenous infusion studies, no equilibrium is reached between plasma and ECF in the brain, but rather a steady state with the concentration in the brain ECF lower than that in plasma, and much closer to that in the CSF [11]. The magnitude of this steady state level depends on the blood brain barrier (BBB) for a certain molecule, the diffusion coefficient in the nervous tissue, the rate of flow of the CSF, and so on.

The low level of compounds in CSF can be produced by at least three possible mechanisms. Firstly, there could be a restriction at the point of entry of the newly formed CSF. Secondly, there could be a process by which these substances are actively transported out of CSF by e.g. the choroid plexuses. Thirdly, the neurons and glia may remove compounds from CSF by active uptake during the passage of this fluid through the ventricular and subarachnoid spaces, via the ECF space.

The mechanisms influencing CSF and brain ECF composition and modes of control will be discussed in the following section, with special emphasis on amino acids, purines and pyrimidines.

The cerebral barriers

The basic modes of fluid and metabolites exchange between blood, CSF, brain ECF and brain parenchyma, are fivefold (see figure 1.1):

(1) a BBB, representing the site of exchange between blood in the CNS capillaries and the ECF;

(2) a blood-CSF barrier, the site of exchanges between blood in the choroid plexuses and the CSF in the ventricles;

(3) relatively free exchange is possible between CSF and brain;

(4) exchange across the cell membranes between ECF and neurons and glia;

(5) exchange across arachnoid membranes is possible between CSF and blood [10].

1.2 The blood-brain barrier

In 1885 Paul Ehrlich described the presence of a diffusion barrier between blood and brain [12]. After injecting laboratory animals with vital dyes he noted that only the brain remained unstained. He believed that the brain had a low affinity for dyes. The reverse experiment was performed by Goldmann in 1913 [13]. He injected dyes into the CSF and observed that only the brain was stained. As a consequence he hypothesized the concept of a BBB. Two barrier systems are present. The BBB is found at more than 99 % of the brain capillaries. The blood-CSF barrier, however, is not found at the small number of capillaries perfusing the choroid plexus (and circumventricular organs as the median eminence, subfornical organ, the area postrema and organum vasculosum of the lamina terminalis cerebri), because these capillaries are fenestrated, but is formed by the tight junctions sealing together adjacent choroid epithelial cells [14]. The surface area of the BBB is 5000-fold greater than that of the blood-CSF barrier, which underscores the quantitative importance of the BBB as compared with the blood-CSF barrier [15].
Electron microscopic studies [14] revealed the specific features of brain capillaries (fig. 1.3). The endothelial tight junctions, the paucity of pinocytosis and the absence of fenestrations in brain capillary endothelia are the primary anatomical characteristics underlying the BBB phenomenon. Pericytes surrounding cerebral endothelia have phagocytic properties, and it has been suggested that the pericyte exercises a barrier function when the capillary integrity is compromised [16]. Evidence has been presented that the astrocytic foot processes induce the formation of barrier-type capillaries [17] (see below).

Next to the anatomical or physical barrier we now recognize an equally important biochemical barrier and regulatory interface functions in the BBB [18]. The biochemical barrier is attributable to a number of enzymes that are largely specific to the brain capillary endothelia. They rapidly degrade certain substrates thus shielding the brain from neuroactive or -toxic effects [19]. For example, noradrenaline and serotonin are inactivated by monoamine oxidase.

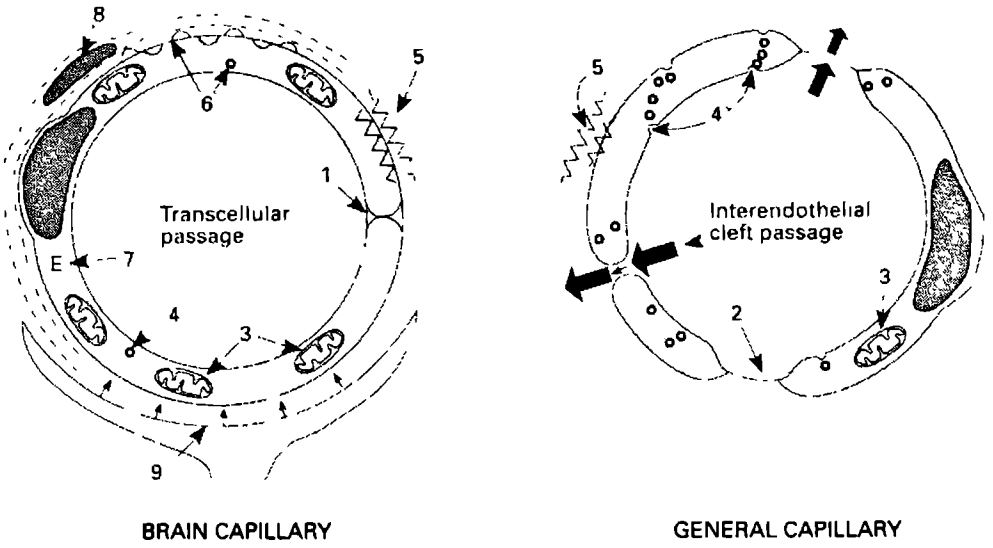

Figure 1.3 *A comparison of features of BBB and periferal tissue capillaries.*

1. *Many capillaries of the BBB are seamless, but cell-to-cell contacts, when observed, are characterized by the presence of* ***tight-junctions.***
2. ***Fenestrations found*** *in other capillaries and also in the choroid plexus capillaries, are absent at the BBB.*
3. ***Higher mitochondrial content*** *at the BBB.*
4. *Little or no* ***pinocytosis*** *occurs at the BBB.*
5. ***Greater electrical resistance*** *imparts low ionic permeability.*
6. *Differences in stucture (proteins) and function of the luminal and abluminal brain capillary surfaces have been established.*
7. ***Enzymes,*** *largely specific to BBB capillary endothelia, rapidly degrade certain substrates.*
8. ***Pericytes,*** *known to have phagocytic properties, serve as the first line of defense if BBB functions are compromized.*
9. *Formation of barrier-type capillaries is induced by a signal from adjacent* ***astrocytes.***

For further explantion see text. Illustration from [20].

The regulatory interface is characterized by:

(a) larger and greater numbers of mitochondria in BBB endothelia, maintaining a low potassium level needed for nerve impuls conduction;

(b) an increased electrical resistance, which is consistent with low ionic permeability;

(c) luminal and antiluminal brain capillary surfaces each having specific functions [18].

These specific functions will be discussed below. In general terms, the BBB provides a homeostatic mechanism by which the unique functions of the brain are maintained.

Development of the BBB

It is not fully understood what causes the induction of the BBB. A role for the glial feet has been suggested in 1972 [21] and now accumulating information [17, 22, 23] indicates that a stimulus from the astrocytic foot processes of the glial cells induces the formation of barrier-type endothelial cells in the central nervous system (CNS). In a study using quail-chick transplantations, it was demonstrated that abdominal host vessels vascularizing grafted neural tissue formed structural, functional and histochemical features of BBB capillaries. The reverse experiment indicated that brain vessels vascularizing grafted mesodermal tissue were devoid of these characteristics [17].

In animal studies the anatomical characteristics of BBB capillaries are observed by the first trimester of fetal life [24], and the BBB of newborn organisms is known to be almost fully developed [25]. Metabolic barrier and regulatory functions have been demonstrated at birth [18]. Moreover, accumulating data suggest that BBB transport mechanisms may operate at much higher rates in the newborn than in the adult brain: e.g. the influx of monocarboxylic acids is higher in suckling brain [26].

BBB transport

Because of the special characteristics of the BBB, transcellular transport is lipid- or carrier-mediated. The brain capillary endothelium allows rapid transit of small-molecule lipophilic compounds, but restricts the penetration of hydrophilic molecules. Compounds, which have little or no affinity for the membrane lipids are preferentially bound to a receptor or transporter protein and translocated to the internal side of the membrane. In this carrier-

mediated, facilitated transport a receptor or transporter specifically recognizes a compound or group of compounds.
This - regulation - system supplies, despite highly variable plasma levels, constant amounts of molecules to the brain.
Seven receptors have been identified [27, 28], and others will be demonstrated. These receptors are believed to act as transcytosis systems and are therefore thought to be present on both luminal and antiluminal surfaces. The most extensively studied is the insulin receptor, where transcytosis is visualized as three sequential steps: endocytosis at the luminal membrane, movement through the endothelial cytoplasm and receptor-mediated exocytosis at the antiluminal membrane, delivering the peptide to the brain interstitium [27, 28].

Transporters or carriers are proteins traversing the capillary membranes which recognize classes of molecules and transfer them from the lumen to the brain in milliseconds, thus accomplishing transit of nutrients. Seven major independent transporters are controlling the brain uptake of: hexoses, monocarboxylic acids, neutral amino acids, basic amino acids, purine bases, nucleosides, and amines such as choline. These transporters, like enzymes, can be kinetically characterized [15, 18, 29], according to the Michaelis-Menten analysis. The half-saturation constants or Km (the concentration of substrate at which one-half of the transporter proteins will be bound by their substrate molecules) of the substrates of these transporter proteins are in the range of normal plasma concentrations [18, 30]. Therefore, mild increases or decreases in the plasma level of a particular substrate will result in comparable increases or decreases in brain influx. Strong increase of e.g. plasma lactate after strenuous exercise will not be reflected in high brain extracellular lactate concentration. In this situation the BBB monocarboxylic acid transporter is over-supplied with substrate and the transporter actually inhibits blood-to-brain transfer, protecting the brain from a potentially harmfull concentration of lactic acid. Thus the transporters function in a manner which promotes brain entry of substrates when plasma concentrations are low, but prevents excess brain influx when plasma substrates reach excessive concentrations. The maximal velocities are a function of both the number of transport proteins in the BBB membranes and the rates at which these proteins mobilize their substrates. Higher maximal velocities are possible with larger amounts of transporter proteins in the BBB; e.g. glucose shows the highest maximal

velocity but also the greatest density of transporters at the BBB, which is consistent with the brain's metabolic requirement for glucose.

Erythrocyte-complemented and protein-complemented BBB transport are concepts which are recently developed [18, 29]. The prevailing view was that plasma-to-tissue exchange was primarily a function of that small free fraction which was not bound to plasma proteins. However, albumin can bind saturably and reversibly to cell membranes, and receptors delaying the transcapillary transit of plasma proteins (and albumin-ligand complexes) could thus effectively complement BBB transport of ligands. It also has been hypothesized that erythrocyte-borne nutrients might be available for BBB transport, but this is more speculative. In that way amino acid and nucleic acid precursors transported into red cells might become available to the brain capillaries [31, 32]. It is possible that circulating peptides may rapidly impart signals to the CNS without traversing the brain endothelia if specific receptors exist, and if a secondary messenger can both receive and transmit the peptide signal [33].

Transport of amino acids across the BBB

There are four transporters involved in amino acid-transport across the BBB. The classification of the carriers is referred to as the Christensen classification [34]. We already mentioned two among the seven major independent transporters, controlling uptake of neutral amino acids and basic amino acids. These are active on the luminal and antiluminal surface of the capillary endothelium, so transport across the BBB involves a single bidirectional system. The first one transports the large neutral amino acids such as phenylalanine, leucine, isoleucine, tryptophane, tyrosine, valine, cysteine, and methionine. This is referred to as the leucine-preferring L-system [34], it is quantitatively the most important carrier. The basic amino acid transport system (the y^+-system) mediates the BBB transport of arginine, lysine, and ornithine [35]. Additionally there are two other transporters, these are only active on the antiluminal surface of the capillary endothelium and they are energy-dependent (ATP-driven) [18]. One is transporting small (non-essential) neutral amino acids [14] such as glycine, alanine, serine and proline, and is referred to as the alanine-preferring A-system. The other one, the X_{AG}-system, is transporting acidic amino acids (glutamate, aspartate) and exports these putative neurotransmitters [36]. Thus these two systems pump amino acids out of the

brain into the capillary lumen. Cooperation between the L- and A-system has been suggested, large neutral amino acids could be transported into brain in exchange for small neutral amino acids. Moreover, in vitro studies have shown that cerebral microvessels take up large neutral amino acids in exchange for glutamine [37].

Competition for transport in an in vitro model system is a classic approach for identifying which amino acids are transported by a given system [34]. The role of competition in vivo, however, has been much less studied. Transport competition effects in vivo are not expected to occur unless the half-saturation constant (Km) of the transport system is low, e.g. about 50-100 μmol/L, which approximates the existing concentration of neutral or basic amino acids in plasma under normal conditions [38]. A survey of amino acid Km values in various tissues shows that the Km of the neutral amino acid transport is in the 1-10 mmol range, except for the BBB where Km values are in the order of 50-100 μmol [36]. The low Km values implicate that availability of a certain amino acid, e.g. phenylalanine, is a function of the plasma concentration and also the concentration of other large neutral amino acids that compete with phenylalanine for transport into the brain. A parameter for predicting phenylalanine availability to the brain is the ratio of serum phenylalanine to the sum of concentrations of competing large neutral amino acids. The low Km of BBB amino acid transport makes the brain uniquely vulnerable to the effects of hyperaminoacidemia. Clinical examples of this are the inborn errors of metabolism: patients with hyperaminoacidemia can show selective defects of the CNS, e.g. mental retardation, seizures, but with little, if any, involvement of other organs [39]. It has been demonstrated in phenylketonuria (PKU), for example, that high circulating phenylalanine levels cause depletions of the brain methionine, isoleucine, leucine, histidine, tryptophane and tyrosine concentration [40, 41]. Experimental hyper-phenylalaninemia in rats resulted in more than a doubling of brain glycine, a result suggesting that inhibitory effects on amino acid exodus may occur also for this tissue [42].

The analysis of Km also leads to insights into the regulation of bidirectional movement of amino acids across the BBB. With the exception of the branched-chain amino acids, there is little net uptake of neutral amino acids by the brain [43]. The overall net uptake of neutral amino acids is only about 10-15 % of the unidirectional flux of amino acids from blood to brain [15]. Therefore, the rate of influx of neutral amino acids from blood to brain is nearly

equaled by the rate of efflux of amino acids from brain to blood. This fact conflicts with the much greater concentration of amino acids in plasma versus brain interstitial space. This rather suggests an active efflux, that contrasts with the idea of a bidirectional and energy-independent L-system. However, the Km on the brain side of the BBB is much lower than the Km on the blood side of the BBB, due to large differences in concentrations of competing amino acids.

Transport of nucleosides and nucleobases across the BBB

The presence of specific saturable transport at the BBB has been demonstrated for certain purines: the adenine carrier transports the purine bases such as adenine, guanine and hypoxanthine [32]. The nucleoside carrier transports the purine nucleosides adenosine, inosine, guanosine and uridine, a pyrimidine nucleoside [32]. There is no known transport system for pyrimidine bases within the BBB, and this is consistent with the ability of the brain to synthesize pyrimidine bases at rates commensurate with its needs [44].
Defects of pyrimidine metabolism should be studied in CSF.

1.3 The blood-CSF barrier and transport of nucleosides, nucleobases and amino acids

Tight junctions sealing together adjacent choroid epithelial cells are the foundation of the blood-CSF barrier. They serve a similar function between the endothelial cells lining the cerebral capillaries, where they create the BBB [45]. These two barriers do not work in the same way, each is specialized to carry specific categories of nutrients into the brain by different mechanisms. The choroid plexus mainly controls the transfer from blood to CSF of some micronutrients which are only needed in the brain in relatively small amounts (e.g. vitamin C, folates, vitamin B6 and deoxyribonucleosides [45]), where the BBB transports substances that the brain consumes rapidly (fig. 1.4). The choroid plexus, the main source of CSF, can also act as an excretory organ by transporting compounds out of the CSF. Ventriculo-cisternal perfusion studies [46] demonstrated an absorptive process against a concentration gradient (active transport). This suggests, that the choroid plexuses may have

an important function in cleansing the CSF [45]. This can be considered as a scavenging activity, regarding for example transmitters and their metabolites [10].

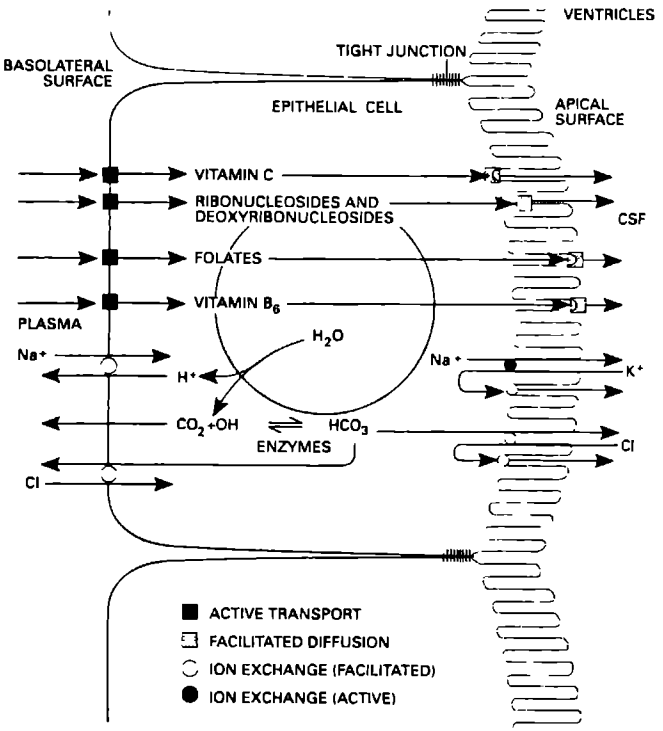

Figure 1.4 *Flow of molecules across the blood-CSF barrier is regulated by several mechanisms in the choroid plexus. Some micronutrients, such as vitamin C, are pulled into the epithelial cells at the basolateral surface by an energy-consuming process known as active transport; the micronutrients are released into the CSF at the apical surface by another regulated process, facilitated diffusion, which requires no energy. Essential ions are also controllably exchanged between the CSF and blood plasma. Transport of an ion in one direction is linked to the transport of a different ion in the opposite direction, as in the exchange of sodium (Na^+) ions for potassium (K^+) ions [45].*

Transport of nucleosides and nucleobases across the blood-CSF barrier

The transport of deoxyribonucleosides, and ribonucleosides, which are different classes of molecules, is governed by a single system in the choroid plexus [45]. Ribonucleosides, but

not deoxyribonucleosides, can also cross the BBB. Both substances compete for acces to the carrier molecules, whichever nucleoside is the most abundant in the blood plasma is transported into the CSF most frequently [45, 47].

Barlin [48] showed that xanthine was accumulated by the in vitro rabbit choroid plexus. Analogues that inhibited the accumulation were hypoxanthine, guanine, adenine, uracil and urate.

From thymidine, required for the de novo synthesis of DNA, it is shown that the concentration in the CSF follows that in the serum quite closely [49]. In adult rats, thymidine crosses the BBB poorly, if at al [32]. Spector [50] considered the possibility that penetration of thymidine into the CSF by way of the choroid plexus might be the primary step in penetration into the brain, as with, for example ascorbic acid. He demonstrated an active transport in choroid plexus [50]. When thymidine was given intraventricularly to rabbits, it left the CSF rapidly by a saturable mechanism, suggesting an active removal [51]. These facts suggest that the choroid plexus may control purine and pyrimidine levels in CSF.

Transport of amino acids across the blood-CSF barrier

The choroid plexuses are capable of actively transporting amino acids from CSF into blood. Davson et al [52] carried out ventriculo-cisternal perfusion of labelled amino acids, simultaneously with intravenous infusion of labelled amino acids, and was able to proof that labelled glycine and methionine continues to be cleared from the perfusion fluid in spite of the large concentration gradient of the different labelled compounds on passing from CSF to blood. Other studies on the isolated choroid plexus showed accumulation of substrates, thus providing another argument for active outward tranport of amino acids [53, 54]. A flux in both directions was demonstrated but no net direction for this movement could be found [53]. Cross-inhibition features in the latter study suggested the involvement of the A-carrier system [54]. The results of Davson's in vivo study [52] broadly agree with these in vitro data showing that there is carrier mediated uptake out of the CSF perfusate. The carrier types broadly follow the Christensen classification [34, 55]. With an indicator dilution method Segal and Zlokovic [56] demonstrated the existence of carriers for the uptake of large neutral amino acids and small neutral amino acids and on the other hand the absence of a significant A-transport system at the blood side of the choroid plexus epithelium. Differences between the possible routes into the CSF, however, have not been cleared up yet. The key question

remains to be answered is whether the concentration of amino acids in the CSF is the result of a restriction at the site of the entry process with the newly formed CSF, or is it due to activity of the choroid plexusus transporting these substances out of the CSF thereby maintaining a steep concentration gradient between this fluid and the neurons of the CNS [56]. A combination of these two mechanisms would also be possible. It is demonstrated that the low level of sugars in CSF is a consequence of the restriction placed on the entry process and not one of active efflux [57]. Whether the same results will hold true for amino acids remains to be seen.

1.4 Exchange between brain ECF and CSF

Since the ependymal lining of the ventricles is highly permeable, it can be seen that the CSF will act as a 'sink' in which the brain ECF amino acids will diffuse, as stated before. So on this site there is no functional barrier.

1.5 Exchange between brain ECF and neurons and glia

The concentration of amino acids in the CSF will depend on the concentration in the plasma and the kinetics of transport across the barriers of the CNS as pointed out above. Particularly important in this respect are exchanges with the central nervous parenchyma since many cells tend to accumulate amino acids in concentrations well beyond the level of their outside medium. The neurons and glia can take up amino acids from the brain ECF, so can exert some control on the composition of amino acids in the ECF. There are 14 or more transport systems present for amino acids in mouse brain tissue [58, 59]. Some of them are high affinity systems, needed for clearing synaptosomes from potent neurotransmitters. Other carriers are active in transporting non-essential amino acids. Five of the carriers described were the A, L, ASc, anionic and kationic systems as described by Christensen [55] and the remainder were specific for glycine, GABA, taurine and lysine. This complexity is necessary for control of these amino acids which also have a neurotransmitter role. Uptake of non-

essential amino acids is very active in brain cells but is mostly absent in brain capillaries where the uptake of essential amino acids is more rapid and several systems seem to be absent (such as for GABA). The K_m and V_{max} values for BBB transport of amino acids are 1/3 and 1/10 of values at the brain cell membrane, respectively [60]. This indicates that transport restriction is located at the BBB and not at the brain cell membrane.
With respect to CSF composition it is also important to realize that the nature of the intracellular cerebral metabolic pathways will influence intracellular fluid (ICF) and ECF composition, and indirect CSF composition, since the availability of certain types of enzymes in brain can be different from other tissues. An example is neurotransmitter metabolism. Another example is the absence of xanthine oxidase in cerebral purine metabolism. This means that uric acid cannot be formed in brain tissue from hypoxanthine. In the following chapters this will be illustrated further.

1.6 Exchange across the arachnoid membranes

The arachnoid membranes have shown to be active in amino acid uptake [61]. The isolated frog arachnoid membrane showed a marked uptake of glycine from CSF to blood [62]. These observations indicate active amino acid uptake by the arachnoid membrane from CSF, the relative importance of this route is not yet known.

1.7 Conclusions and aim of study

In this introducing chapter a type of system is described, with a restricted entry at the BBB and blood-CSF barrier, active uptake by neurons and a bulk flow drainage into CSF, and clearance from the CSF by choroid plexus and the arachnoid membrane. This system could act as an efficient homeostatic mechanism for the brain ECF amino acid, purine and pyrimidine control.
The acces to this system in the clinical setting is by way of CSF examination. This route has become more important since the biochemical basis of most metabolic or supposed metabolic

brain diseases in children remains unknown. Metabolic brain disease can be associated with abnormal CSF levels of purines, pyrimidines, or amino acids. The availability of new and more sensitive methods for determining these compounds urged us to establish normal values of amino acids, purines and pyrimidines in CSF. Our ultimate aim was to study CSF amino acid, purine and pyrimidine levels in various neurological disorders hoping to contribute to the etiologic unraveling of metabolic brain disease. Moreover, we wanted to answer the question whether the determination of the concentration of amino acids, purines and pyrimidines in CSF is a usefull tool in screening for metabolic disorders in children with unknown psychomotor retardation. The following chapters describe the results of our efforts.

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Chapter 2

REFERENCE VALUES FOR NUCLEOSIDES AND NUCLEOBASES IN CEREBROSPINAL FLUID OF CHILDREN

Chapter 2

Reference values for nucleosides and nucleobases in cerebrospinal fluid of children

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Key words: pediatric chemistry, heritable disorders, metabolic disease, sex- and age-related effects, liquid chromatography, uridine, uric acid, neurological disorders, purines, pyrimidines

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2.1 Abstract

Disturbances in the metabolism of purines and pyrimidines in neurologically affected patients can be reflected by aberrant concentrations of nucleosides and nucleobases in cerebrospinal fluid (CSF). However, normal values, especially for children at different ages, are lacking. We collected 1000 specimens of CSF from subjects ranging in age from newborn to 18 years, who were undergoing a diagnostic lumbar puncture for several clinical indications. Of these, 78 samples could be used retrospectively as a reference according to our criteria. The analyses were performed with a modified HPLC procedure. None of the substances shows age-dependency except uridine and uric acid. Uridine increases with age, and uric acid increases with age in boys older than 12 years.

2.2 Introduction

Purine and pyrimidine bases and nucleosides in biological fluids can now be measured with sufficient sensitivity by 'high-performance' liquid chromatography (HPLC) [1, 2]. However, data on the concentrations of nucleobases and nucleosides in human cerebrospinal fluid (CSF) of children are lacking, although some reports have been published about the oxypurines in CSF in relation to hypoxia and cerebral ischemia [3-6].

Abnormal concentrations of some purine and pyrimidine bases in CSF have been reported in a few cases of deficiencies in purine and pyrimidine metabolism. Defects have been described for the *de novo* synthesis pathway, for nucleotide interconversion, for the salvage pathway, and for purine and pyrimidine catabolism [7-10]. Subjects with these disorders frequently present with neurological symptoms and psychomotor retardation.

We measured the concentrations of nucleobases and nucleosides in CSF of children of different ages. The data we report here can be used as reference values for further studies of purine and pyrimidine metabolism in relation to brain function and to various neurological disorders.

2.3 Materials and methods

Samples

From 1983 to 1987, 1000 specimens of CSF were obtained from subjects ranging in age from three days to 18 years, who were undergoing a diagnostic lumbar puncture for conventional clinical indications such as suspected central nervous system infection or a neurological disorder.

Whenever possible, 1 ml of each CSF sample was put aside for the present investigation. We were able to use 78 of the samples, selected according to the following criteria: no evidence of a neurological disorder, of inherited metabolic disease, or of malignant disease, and erythrocyte content $< 100/\mu l$. Figure 2.1 shows the age distribution of these 78 subjects.

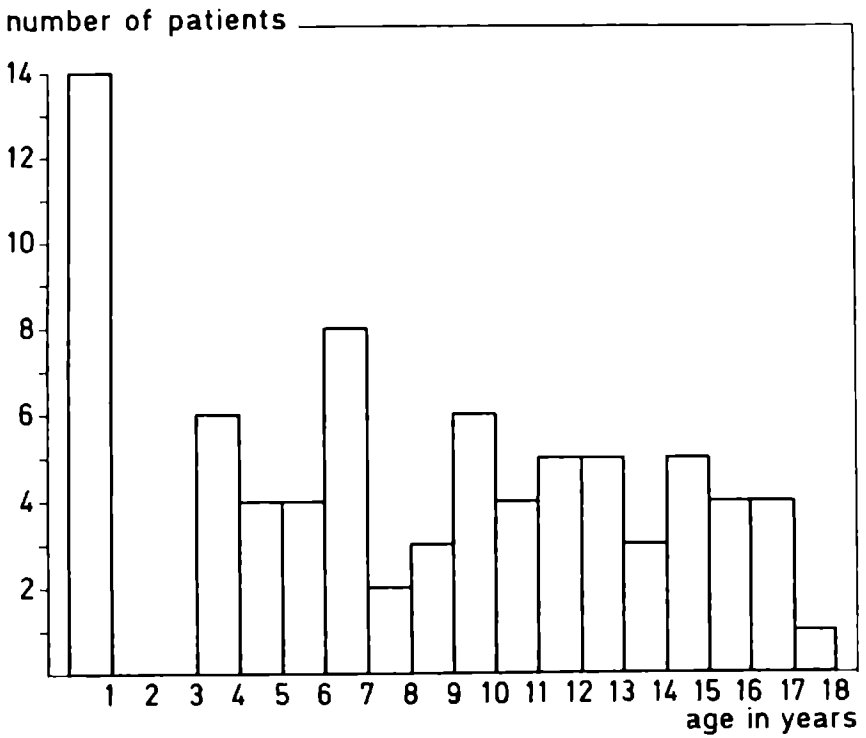

Figure 2.1 *Age distribution of subjects.*
Group 1 (infants, ages 3 days to 12 months) consisted of 6 boys and 8 girls.
The remaining 64 subjects (ages 3-18 years) consisted of 40 boys and 24 girls.

Extraction procedure

The CSF samples were stored at -70° C until analysis. Just before analysis the samples were thawed, then deproteinized by adding 8 mol/l perchloric acid at 1/20 of the sample volume. After mixing, the samples were kept in crushed ice for 20 min and then centrifuged (9000 x *g*) for 5 min at room temperature. We precipitated the excess perchloric acid in the cold as potassium perchlorate by adding a mixture containing 1 mol of potassium hydroxide and 4 mol of dipotassium hydrogen phosphate per liter (this adjusted the pH range of the sample to 6.0-6.7). The volume of the mixture was approximately a tenth the volume of perchloric acid extract. After 15 min on ice, the neutralized extract was again centrifuged. We used the supernate for HPLC analysis.

HPLC procedure

For HPLC we used a Model SP 8000B liquid chromatograph (Spectra Physics, Santa Clara, CA), connected to a cooled (4° C) automatic sampler (MSI660; Kontron, Electrolab, London, U.K.), and 250 x 4.6 mm columns of Supelcosil (5-μm particle size; Supelchem BV, Leusden, The Netherlands). Column temperature was 35° C, flow-rate was 0.7 ml/min, and the sample loop delivered 200 μl. Table 2.1 lists the mobile phase components and their proportions. All mobile phases were degassed by continuous purging with helium. Before use, we filtered the solutions through a Millipore filter (type HA, pore size 0.45 μm; Millipore Corp., Bedford, MA).

Table 2.1 *Composition of the Mobile Phase Used in the Modified HPLC procedure*

assay	KH_2PO_4, 0.1 mol/l pH 4.5	Methanol, 500 ml/l	Distilled water
time, min		% of total volume	
0	98	2	0
10	40	5	55
20	40	7	53
30	40	7	53
45	5	30	65

The indicated mixtures are reached at the indicated time intervals

We monitored the column effluents at 254 and 280 nm, calculating concentrations from the peak areas at 254 nm. Comparisons were made with external standards included in the beginning of the run and periodically between the samples.
Figure 2.2 shows a characteristic chromatogram of nucleosides and nucleobases.
The minimum detectable concentrations of several purines and pyrimidines (μmol/l) were: hypoxanthine and guanosine 0.01; cytidine, thymine and xanthine 0.04; and uric acid 0.08.

Statistics

Most substances displayed a gaussian distribution; we calculated their reference limits as the mean value $\pm$ 1.96 SD. For those substances for which more than half of the samples had values below the detection limit, we calculated sample-based upper percentiles (95 and 97.5 percentiles) for the older children. Age and sex dependency of values was investigated by regression analysis. Results for groups I (3 days-12 months) and II (3-18 years) were compared by Student's *t*-test. We had no subjects between 12 and 36 months of age.
The number of patients in group I is small, and we present their data only for the sake of comparison.

2.4 Results

Table 2.2 lists the reference values for the purine and pyrimidine bases and nucleosides in CSF of children. The results of the analysis of CSF of these 'normal' children show that two clusters of substances can be distinguished. One is usually detectable in measurable amounts and includes inosine, hypoxanthine, xanthine, uric acid, guanosine, uridine, uracil and cytidine. A second cluster of substances, only sometimes detectable, includes adenine, adenosine and thymine.
In some cases cytidine, uric acid and uracil were difficult to measure because of insufficient separation. Therefore fewer values are reported for them.
When comparing groups I (infants of age 3 days to 12 months) and II (children, ages 3-18 years) we saw no statistically significant differences in reference values for most of the nucleobases and nucleocides, except for inosine, which is lower in infants.

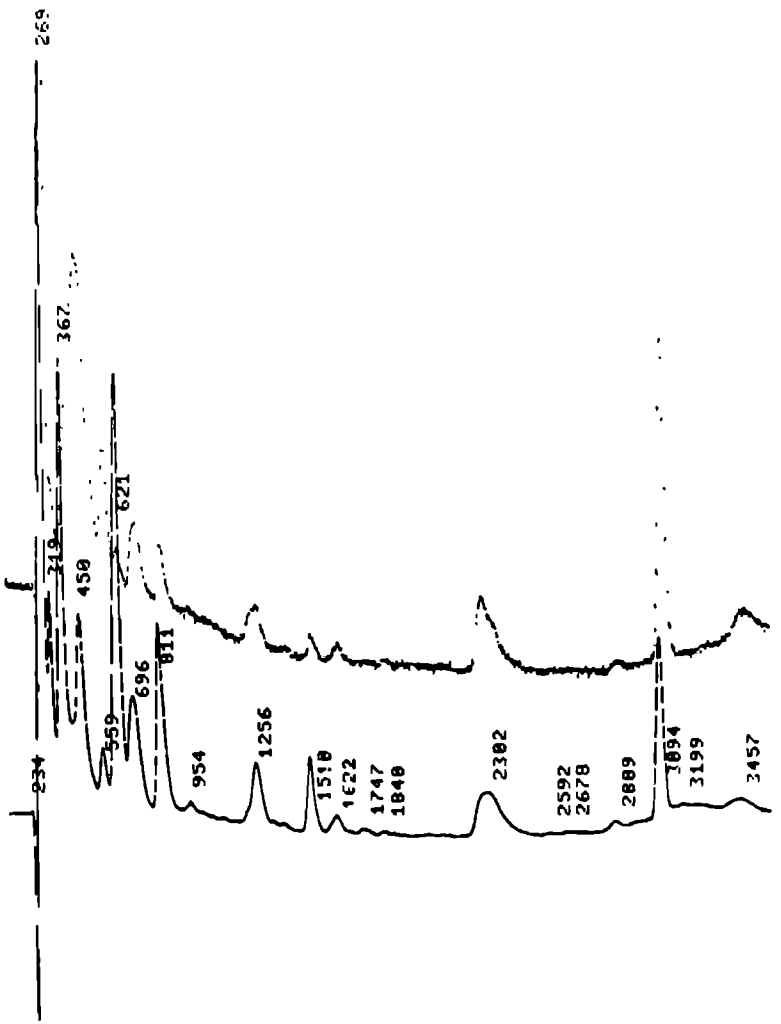

Figure 2.2 *A characteristic chromatogram of nucleosides and nucleobases in a CSF sample.*

Compound	*Retention time, s*	*Compound*	*Retention time, s*
Uracil	*319*	*Xanthine*	*696*
Pseudouridine	*367*	*Uridine*	*811*
Uric acid	*450*	*Thymine*	*954*
Cytidine	*559*	*Inosine*	*1510*
Hypoxanthine	*621*	*Guanosine*	*1622*

Table 2.2 Concentrations (μmol/l) of Nucleobases and Nucleosides in CSF from healthy infants and children

	infants (n = 14, ages 3 days to 12 months)				children (n = 64, ages 3-18 years)			
	n	mean	SD	mean ± 1.96 SD	n	mean	SD	mean ± 1.96 SD
Inosine	14	0.37	0.11	0.15-0.59	64	0.50	0.19	0.14-0.86
Hypoxanthine	14	2.38	0.89	0 64-4.12	64	2.47	0 71	1.07-3.87
Xanthine	14	2.08	1.06	0-4.16	64	1.73	0.62	0.51-2.96
Uric acid	9	16.32	18.14	0-51.87	55	10.22	6.78	0-23.51
Guanosine	14(4)[a]	0.10	0.13	0-0.35	64(4)	0.12	0.09	0-0.31
Uridine	14	2.02	0.86	0 33-3.71	64	2.12	0.59	0.96-3.28
Uracil	7	1.25	0.55	0.17-2.33	31(4)	1.12	0.77	0-2.62
Cytidine	7(1)	0.44	0.32	0-1.07	49(6)	0.34	0.20	0-0.72
Adenine	14(6)	0.24[b]			64(46)	0.37/0.59[c]		
Adenosine	14(14)	<0.02[b]			64(57)	0.54/0.98[c]		
Thymine	14(7)	1.05[b]			64(42)	0.31/0.93[c]		

[a] Number of subjects with concentrations below the detection limit given in parentheses.
[b] Maximum concentrations measured.
[c] 95th/97.5th percentiles of measurable concentrations.
Values for uridine and uric acid for a specific age can be calculated with the formulas presented in Figs. 3 and 4.

In children, none of the substances showed age-dependency except uridine and uric acid. Uridine concentration increases with age (fig. 2.3). Uric acid increases with age in boys older than 12 years; in this case there is also a sex-related difference (fig. 2.4).

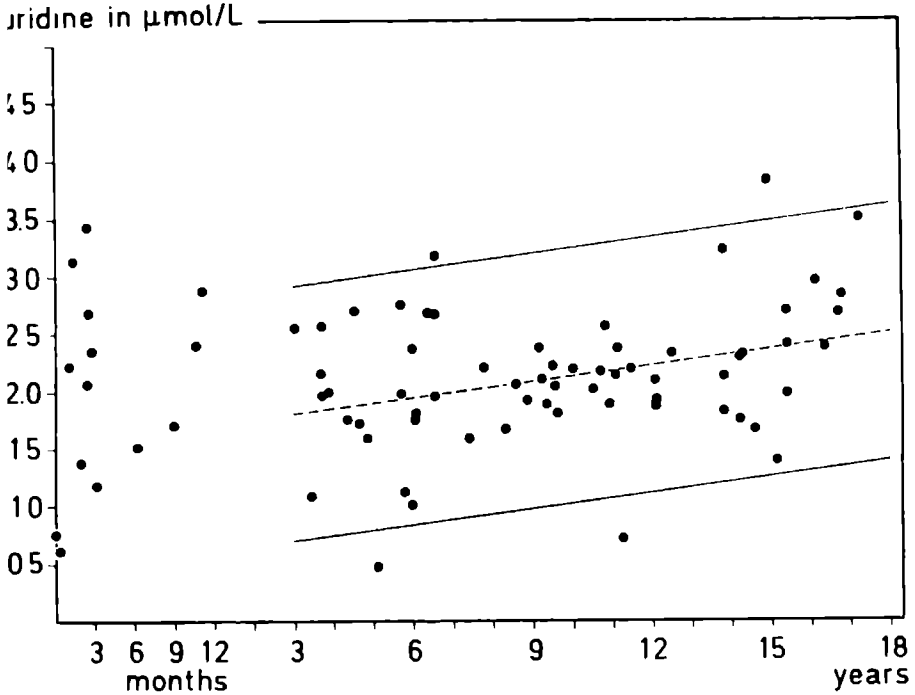

Figure 2.3 *Changes in concentrations of uridine with age*
By linear regression, uridine (µmol/l) (± SD) = 1.67 + [0.046 x age (years) (± 0.56)]
The lines indicate mean ± 1.96 SD.

Although the number of specimens analyzed for uric acid in group I is small, there seems to be an age-related difference between the patients in group I and II.
Three children had remakably high values for certain analytes. For example, a three-month-old boy, the healthy brother of a child who died because of sudden infant-death syndrome, had high values for thymine (1.05 µmol/l). Other extensive examinations did not reveal any abnormalities.

Two children of age 7½ and 10 years showed high values for adenosine (0.67 and 1.24 μmol/l, respectively); their only complaint was headache and all other test results were normal. The values for all the other components measured were within normal limits in these children.

We also looked for correlations between metabolically related substances. For group II, the Pearson correlation coefficient between hypoxanthine and xanthine was 0.49, between hypoxanthine and inosine 0.33, and between inosine and guanosine 0.38.

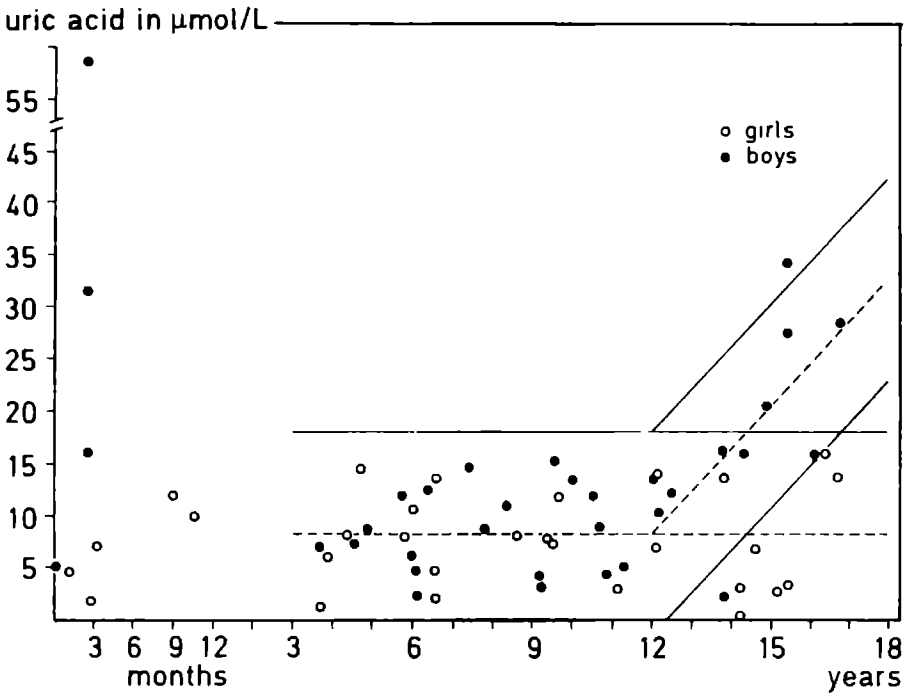

Figure 2.4 *Sex- and age-related differences in uric acid concentrations*
For girls and for boys ≤ 12 years, mean (± SD) concentration is 8.35 (± 4.94) μmol/l. For boys > 12 years, the concentration is described by the following linear regression equation: concentration (μmol/l) (± SD) = 8.35 μmol/l + 4.07 [age (years) - 12] (± 4.94). The lines indicate mean ± 1.96 SD.

2.5 Discussion

Here we compare our results with those already reported in the literature, and comment on some of the striking results.

Table 2.3 summarizes most of the reported data. With respect to the oxypurines it appears that our reference values for hypoxanthine and xanthine in children compare well with those found in adult controls by Eells and Spector [11] and Hällgren et al. [6]. Harkness and Lund [5] obtained higher values for younger infants. Inosine values in our group I have a smaller range than we found in group II or was found by Harkness and Lund [5].

Uric acid values have wide ranges in all studies. In children we found reference values with a mean value of 10.22 μmol/l, comparable with concentrations in CSF reported by Hällgren et al. [6]. Eells and Spector [11] reported higher control values in a group of 10 adults. From the age of 12 years we found a sex-related difference, boys having higher values than girls (fig. 2.4). After puberty, boys also have higher values than girls for uric acid in serum [16]. Hällgren et al. [6] did not find such a difference in adults.

Uric acid can be regarded as a sensitive marker of blood-CSF barrier function, being a low-molucular-mass substance that cannot be formed in cerebral tissue owing to the lack of xanthine oxidase (EC 1.1.3.22) in the brain [12]. This might explain its greater range in infancy as compared with the other nucleobases and nucleosides in CSF, because of the different rates of maturity of the blood-CSF barrier in the young. Values for uridine in CSF in our reference group of children compare well with control values in adults as reported by others [5, 11]. It was the only substance in our study that showed a linear increase with age, after the age of three years (fig. 2.3).

In 29 normal children we also measured the concentrations of uridine in plasma, finding a similar increase with age as was found for CSF. Spector [14] described the choroid plexus as having an active-transport system from blood into choroid plexus and an efflux system for nucleosides out of choroid plexus into CSF. Our observations could be explained by these mechanisms.

Table 2.3 *Our results compared with those in the literature (μmol/l, given in mean ± SD)*

Source	14 infants 3 days-12 months this study	10 infants ref. 5	64 children 3-18 years, this study	10 adults, ref. 11	26 adults, ref. 6	29 adults, ref. 5
Inosine	0.37 ± 0.11	0.7 ± 0.7	0.50 ± 0.19	0.7 ± 0.22		0.2 (0-1.0)[a]
Hypoxanthine	2.38 ± 0.89	3.6 ± 0.9	2.47 ± 0.71	2.5 ± 1.12	2.6 ± 0.5	1.8 (0.6-5.1)
Xanthine	2.08 ± 1.06	5 ± 2.0	1.73 ± 0.62	2.3 ± 0.45	1.7 ± 0.4	1.7 (0.6-4.7)
Uric acid	16.32 ± 18.14	30 ± 25.0	10.22 ± 6.78	24.0 ± 8.74	12.8 ± 9.8	
Uridine	2.02 ± 0.86	3.3 ± 1.4	2.12 ± 0.59	2.3 ± 0.90		1.6 (0.3-8.2)
Cytidine	0.44 ± 0.32		0.34 ± 0.20	0.2 ± 0.0		

[a] Geometric means.

As far as the other nucleobases and nucleosides are concerned, we could find no reliable CSF reference values in the literature. Only Eells and Spector [11] studied these substances in CSF, but they could not detect measurable amounts of cytidine, uracil, thymine, adenine or adenosine. In contrast, we found cytidine, uracil and guanosine in very low but measurable concentrations. Thymine, adenine and adenosine were detectable only in some cases.
Not enough reference values for nucleobases and nucleosides are yet available for infants between one and three years. Our values for the ages three days to 12 months and for three years and older allow a cautious interpolation of values for infants between one and three years.
In this study we obtained reference values for purines and pyrimidines in children of different ages. The availability of these values allows interpretation of CSF analyses of patients with different pathological conditions such as psychomotor retardation, epilepsy, CNS infections and other neurological disorders - perhaps leading to a better understanding of the role of purines and pyrimidines in CNS metabolism.

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Chapter 3

REFERENCE VALUES FOR AMINO ACIDS IN CEREBROSPINAL FLUID OF CHILDREN DETERMINED USING ION-EXCHANGE CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

Chapter 3

Reference values for amino acids in cerebrospinal fluid of children determined using ion-exchange chromatography with fluorimetric detection

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3.1 Summary

One thousand specimens of CSF were collected from subjects ranging in age from newborn to 18 years, who were undergoing a diagnostic lumbar puncture. Sixty-two samples were judged retrospectively as being suitable for calculating age-related reference values. The analyses were performed by an amino acid analyser using ion-exchange chromatography with fluorimetric detection giving a tenfold increase in sensitivity, thereby enhancing the diagnostic capabilities. As many as 36 known compounds could be detected, 10 peaks could not be identified. In children older than 3 years nine of the identified compounds showed age-dependency. We found 22 amino acids to be significantly higher in infants younger than 1 year, with only γ-aminobutyric acid being significantly lower in infants. Alpha-aminoadipic acid showed a sex difference, being slightly higher in girls.

3.2 Introduction

Several investigators have reported on amino acids in CSF of normal subjects [1-9] and in various neurological disorders [1, 9-12]. Limited information is available about normal values of amino acids in CSF in infancy [2, 9, 11] and childhood. Establishing these values is difficult: CSF aliquots of controls can hardly be obtained in childhood and these samples are often inadequately prepared as previously observed [13-14]. Application of a new, more sensitive, method for measurement formed an additional reason for determining reference values of amino acids in CSF in childhood. The concentrations of amino acids in CSF of 62 'normal' children at different ages (3 days-18 years) were measured by ion-exchange chromatography with fluorimetric detection. This method has a much higher sensitivity than the conventional one using ninhydrin-detection. Using the latter Honda [9] found 17 compounds whilst Heiblim et al. [11] found 25 compounds. We were able, however, to measure accurately 36 known compounds including GABA and in addition to detect 10 unidentified peaks.

The aim of this study is to present extended reference values in infants and children. Age- and sex-dependency have been studied too. The data presented are compared with those in the literature and will be used in further studies investigating amino acid metabolism in relation to brain function in various neurological disorders.

3.3 Materials and methods

Samples

From 1983 to 1987 1000 specimens of CSF were obtained from patients ranging in age from 3 days to 18 years, who were undergoing a diagnostic lumbar puncture during the investigation of suspected meningitis or neurological disorders.

Whenever possible, 1 ml of each CSF sample was put aside for the present investigation. We were able to use 62 samples for compiling reference intervals according to previously published criteria [15]: where there existed no evidence of a neurological disorder, of inherited metabolic disease, or of malignant disease, and erythrocyte content < 100 /μl, the spinal fluid showed a normal protein content and the number of white blood cells was normal.

Sample preparation

The CSF samples were kept frozen at -70° C until analysis. Just before analysis the samples were thawed, then deproteinized by adding 0.05 ml of a 3 mol/l sulfosalicylic acid solution to 1 ml of the sample. The sulfosalicylic acid solution contains 180 μmol/l norleucine as an external standard. After mixing, the samples were kept on ice for 10 min, then centrifuged (3500 x g) for 10 min at room temperature. The supernatant was centrifuged again for 10 minutes. We used 250 μl of the supernatant for analysis.

Amino acid analysis

Amino acids in CSF were determined using ion-exchange chromatography on an amino acid analyser (Biotronik LC 6000 or LC 6001, Biotronic, Frankfurt, FRG) according to the procedure advocated by the manufacturer with some modifications.

In order to increase the sensitivity of the method, the ninhydrin detection system was replaced by a fluorescence detection system using o-phthaldialdehyde (OPA) [16] as a reagent. A tenfold increase in sensitivity was obtained employing a Biotronik-fluorescence detector (BT 6630). Reaction time was reduced to 1.5 min and reaction temperature was changed from 100°C to room temperature. The column eluate and the reagent were mixed at a ratio 1:1 (35 $ml \cdot h^{-1}$ each). To prepare buffer solutions lithium chloride was replaced by lithium citrate$\cdot 4H_2O$. The same final lithium concentration was achieved as in the original procedure using ninhydrin. This modification was introduced because of impurities in the commercially available lithium chloride. Phenol in the buffer solution was also replaced by caprylic acid (0.1 $ml \cdot l^{-1}$).

Statistics

Amino acids concentrations displayed lognormal distributions, so reference intervals were calculated after logarithmic transformation. Age (children above 3 years) and sex-dependency were investigated by linear regression analysis applied on the logarithmically transformed data. A separate comparison was made between infants and children by a t-test (logtransformed data).

3.4 Results

The controls were subdivided into two age groups: group I, infants aged 3 days-12 months, and group II, children aged 3-18 years. Not enough samples could be collected between 1 and 3 years. The reference values for the different compounds for both groups are given in table 3.1. A few compounds are not always detectable or only in trace amounts because of their low concentration. These compounds are: cystine, cystathionine, ß-alanine, hydroxylysine, 1-methylhistidine and homoarginine. Their values are given in table 3.2. In group I (infants ages 3 days-12 months) carnosine could be measured only four times.

Table 3.1 *Concentrations (μmol/l) of amino acids in CSF from healthy infants and children*

Amino acids	infants (n = 12, ages 3 days to 12 months)					children (n = 50, ages 3-18 years)					
	mean (SD)		P2.5 *	P50	P97.5	age ** in years	mean (SD)		P2.5	P50	P97.5
taurine	8.76	(2.28)	5.32	8.52	13.65	3-18	6.15	(1.21)	4.05	6.03	8.98
phosphoethanolamine	4.62	(0.96)	3.05	4.53	6.74	3-18	4.87	(1.09)	3.20	4.77	7.11
aspartic acid	6.45	(1.70)	3.70	6.24	10.52	3-18	3.08	(0.58)	2.08	3.03	4.39
threonine	50.04	(25.26)	15.09	44.32	130.17	3-18	24.91	(7.31)	13.99	23.98	41.11
						3			12.78	21.02	34.57
						10			15.06	24.78	40.75
						17			17.76	29.21	48.05
serine	51.96	(12.31)	29.57	30.39	85.89	3-18	30.91	(5.54)	21.17	30.41	43.67
asparagine	8.98	(2.76)	4.46	8.56	16.42	3-18	5.04	(1.16)	3.23	4.92	7.50
						3			2.93	4.54	7.04
						10			3.24	5.02	7.78
						17			3.58	5.55	8.61
glutamic acid	3.45	(2.18)	0.79	2.89	10.56	3-18	4.13	(2.09)	1.21	3.60	10.71
						3			0.67	2.55	6.55
						10			1.26	3.80	9.19
						17			2.05	5.48	12.76
glutamine	574.07	(105.52)	387.59	565.00	823.60	3-18	495.96	(81.24)	352.27	489.33	679.73
α-aminoadipic acid	0.56	(0.23)	0.23	0.52	1.18	3-18	0.57	(0.30)	0.19	0.50	1.34
glycine	5.61	(0.97)	3.82	5.52	7.98	3-18	5.39	(1.26)	3.33	5.25	8.27
alanine	26.56	(5.04)	16.49	26.00	40.98	3-18	20.34	(4.63)	12.64	19.82	31.10
						3			11.67	17.54	26.36
						10			13.40	20.13	30.25
						17			15.37	23.10	34.71
citrulline	3.55	(2.45)	1.25	3.12	7.80	3-18	1.65	(0.36)	1.06	1.62	2.46
α-aminobutyric acid	2.94	(0.67)	1.95	2.88	4.25	3-18	2.48	(1.16)	0.97	2.26	5.26

valine	19.18	(4.76)	11.91	18.70	29.37	3-18	12.84	(2.60)	8.70	12.61	18.28
						3			7.60	11.01	15.94
						10			8.84	12.80	18.53
						17			10.27	14.88	21.55
methionine	3.36	(1.31)	1.55	3.40	7.44	3-18	2.20	(0.65)	1.15	2.10	3.83
						3			0.93	1.68	3.03
						10			1.19	2.14	3.86
						17			1.52	2.74	4.93
isoleucine	7.58	(1.86)	4.66	7.38	11.71	3-18	4.16	(1.00)	2.53	4.04	6.46
leucine	15.68	(1.81)	12.39	15.58	19.59	3-18	10.38	(2.51)	6.51	10.11	15.70
						3			5.63	8.65	13.31
						10			6.75	10.38	15.96
						17			8.09	12.44	19.13
tyrosine	13.55	(3.42)	6.86	13.01	24.67	3-18	7.99	(1.87)	4.69	7.75	12.80
phenylalanine	11.60	(5.51)	5.15	10.77	22.50	3-18	8.18	(3.85)	4.32	7.72	13.79
γ-aminobutyric acid	0.05	(0.04)	0	0.05	0.13	3-18	0.31	(0.14)	0.05	0.30	0.61
ethanolamine	12.07	(7.14)	3.35	10.34	31.86	3-18	15.78	(4.00)	9.10	15.28	25.65
tryptophane	4.16	(1.47)	2.10	3.96	7.47	3-18	2.16	(0.60)	1.06	2.06	3.98
ornithine	8.17	(3.75)	2.84	7.38	19.17	3-18	3.95	(0.96)	2.42	3.84	6.10
lysine	21.38	(6.13)	11.61	20.57	36.47	3-18	17.25	(4.10)	10.00	16.72	27.97
						3			9.56	14.29	21.37
						10			11.53	17.24	25.78
						17			13.91	20.80	31.10
histidine	18.36	(5.05)	10.46	17.75	30.12	3-18	13.18	(2.59)	8.73	12.93	19.15
n-ε-methyl-l-lysine	3.28	(1.12)	1.64	3.12	5.94	3-18	2.18	(1.77)	0.36	1.61	7.29
3-methyl-histidine	0.48	(0.30)	0.14	0.42	1.21	3-18	0.38	(0.21)	0.16	0.35	0.76
homocarnosine	8.23	(1.77)	5.54	8.08	11.79	3-18	5.44	(1.86)	2.47	5.12	10.59
						3			3.19	6.33	12.58
						10			2.47	4.91	9.76
						17			1.92	3.81	7.57
arginine	20.00	(4.96)	11.92	19.44	31.70	3-18	20.41	(4.56	12.82	19.92	30.96

* P2.5, P50 and P97.5 according the lognormal model.

** Values for the ages 3, 10 and 17 years were calculated according to linear regression analysis.

Table 3.2 *Amino acids which are not always detectable; their frequence of absence and the number of healthy infants and children in which we were able to quantify the concentrations in CSF (in µmol/l).*

Compound	Group I (n = 12)			Group II (n = 50)		
	absent (n)	quantified (n)	concentrations	absent (n)	quantified (n)	concentrations
cystine	9	2	0.15, 3.93	33	5	0.2, 0.31, 0.33, 0.6, 1.04
cystathionine	3	0		18	5	0.12, 0.37, 0.5, 0.64, 2.36
alanine	3	6	0.06, 0.1, 0.18, 0.24, 0.29, 0.42	44	1	0.07
hydroxylysine	1	10	0.05, 0.07, 0.08, 0.11, 0.19, 0.2, 0.4, 0.58, 2.42, 2.57	23	6	0.05, 0.08, 0.11, 0.15, 0.78, 4.06
1 methyl-histidine	5	0		7	10	0.06, 0.07, 0.09, 0.11, 0.12, 0.16, 0.83, 0.87, 1.03, 1.14
homoarginine	12	0		16	2	0.46, 0.59

When comparing group I and II, there are some significant age dependent differences in reference values. Taurine, aspartate, threonine, serine, asparagine, glutamine, alanine, citrulline, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ß-alanine, ethanolamine, tryptophane, ornithine, lysine, histidine, n-ε-methyl-1-lysine and homocarnosine are significantly higher in infants (all $p < 0.001$, except glutamine: $p = 0.028$, phenylalanine: $p = 0.002$, ethanolamine: $p = 0.004$, n-ε-methyl-1-lysine: $p = 0.005$ and homocarnosine: $p = 0.038$). Gamma-aminobutyric acid (GABA) is the only compound which is significantly lower in infants ($p < 0.001$) (fig. 3.1). The other amino acids show no difference between both groups.

Within group II, nine amino acids show an age-dependency in childhood. Threonine, asparagine, glutamate, alanine, valine, methionine, leucine and lysine increase with age (all $p \leq 0.01$, except asparagine: $p = 0.043$). Homocarnosine is the only compound measured which decreases with age ($p = 0.015$) (fig. 3.2). In none of the substances we find any sex differences, except for α-aminoadipic acid where females have slightly higher values than males ($p = 0.047$).

We found 10 hitherto unidentified compounds. In group I, 4 and in group II, 6 of these are always present.

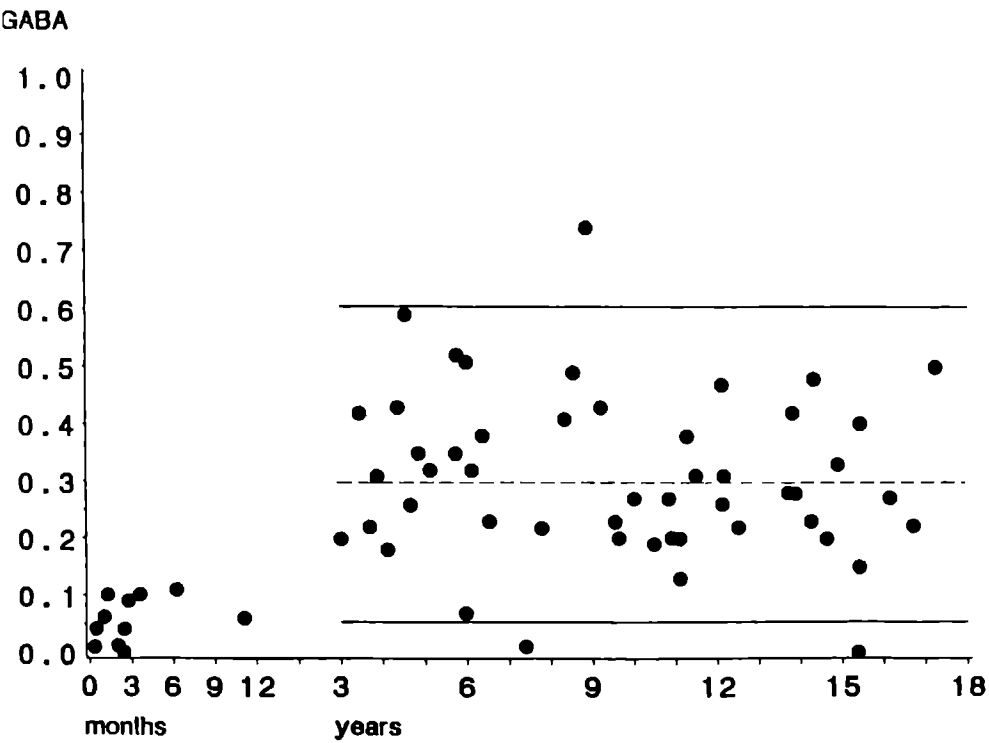

Figure 3.1 *Concentrations of free γ-aminobutyric acid in CSF as a function of age (μmol/l). The lines indicate mean, p 2.5 and p 97.5.*

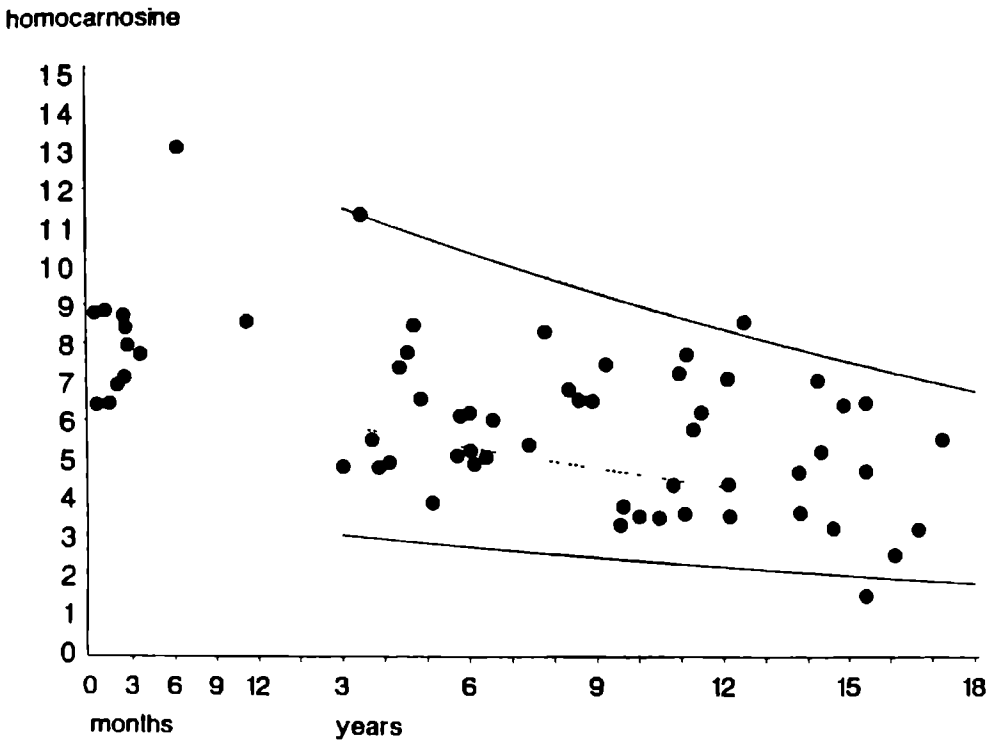

Figure 3.2 *Concentrations of homocarnosine in CSF as a function of age (μmol/l). The lines indicate mean, p 2.5 and p 97.5.*

3.5 Discussion

Normal values for amino acids in CSF are only rarely reported in literature, especially for young infants and children [2, 9, 11]. When published, these data have been collected from measurements using ion-exchange chromatography with the ninhydrine detection. Due to low concentrations of amino acids in CSF compared with serum, the sensitivity of this method is inadequate to measure accurately the concentration of those amino acids which are normally present in low amounts, e.g., GABA, homocarnosine, α-aminoadipic acid, ß-alanine, hydroxylysine and 3-methylhistidine. A few investigators have used

HPLC procedures with fluorimetric detection to characterize CSF amino acid profiles [3, 17]. This method, has a lower resolution, however, than our technique. These considerations prompted us to study the amino acid cerebrospinal fluid concentrations in a large group of children of different ages using a highly sensitive detection system involving fluorimetric detection after post-column derivatization with o-phthaldialdehyde. Using this method we were able to measure more reliable the concentrations of a greater number of amino acids than previously reported, to include particularly compounds of special importance in cerebral metabolism like GABA and homocarnosine. In addition, 10 unidentified compounds could be detected using this fluorimetric detection. In the near future attempts will be made to identify these compounds in order to increase the diagnostic potential of the procedure. Three of the unidentified compounds appear less frequently in infants which could be a reflection of cerebral immaturaty.

We found the concentration of 22 amino acids to be higher in younger children. Liappis et al. [5] found in the youngest age group (1-24 months) higher values for 7 amino acids in boys but only serine in the case of girls when compared with the older age group (2-14 years). In our study only GABA was lower in younger children.

Using the present method we were able to measure accurately the concentration of GABA. Until now there have been only a few well documented studies on the determination of this very important inhibitory neurotransmitter [3, 17-20]. For young infants (0-12 months) we found similar values for GABA to those determined by Goldsmith et al. [19], whereas higher values were found by us for the older children. Goldsmith et al. [19] used a method based on reverse-phase liquid-chromatography with precolumn derivatization with OPA. They did not, however, deproteinize their samples because of the possible breakdown of conjugated GABA during deproteinization with sulfosalicylic acid. In spite of involvement of this deproteinization step in our procedure, we recorded comparable values for the youngest children. As far as age-dependency is concerned we can confirm the findings of Goldsmith et al. [19] about GABA concentration as being lower in children younger than 1 year. Hare et al. [20] found a downward trend with age in adult females. The higher GABA concentration in our group II as compared with group I could be related to an increase of the activity of the primary GABA synthetic enzyme, glutamic acid decarboxylase (GAD) (EC 4.1.1.15), during early life. A tenfold increase of this enzyme during the first month of life has been established

in studies on developing rat brain [21,22], which is consistent with Purkinje cell maturation in the cerebellum and maturation of the GABA-ergic system.

Little is known on age-dependency of amino acids in CSF of older children. As stated, we found a positive correlation with age for 8 amino acids. McGale [23] reported a positive correlation with age for only valine and glycine. His patients, however, aged 10-69 years, had diffuse neurological syndromes.

Within our group II, homocarnosine was the only compound which decreased with age. Perry et al. [7,24] found much higher values for homocarnosine in infants and children than in adults. Van Sande et al. [1] reported a significant decreasing trend with age for homocarnosine. Takahashi [25] reported a rapid increase of homocarnosine in CSF after birth until two years of age. Between 2 and 8 years the homocarnosine level remains constant and after 9 years the homocarnosine level decreases. Homocarnosine is present only in the central nervous system. Increased homocarnosine concentrations may occur in parallel with functional alteration of brain, especially of the cerebellum [25]. Several studies [13, 25-31] reported on the GABA-containing dipeptide homocarnosine (γ-aminobutyrylhistidine) and the related precursor GABA. Despite this relationship we could not find a correlation in the present study.

No sex differences were found in both groups, except for α-aminoadipic acid, being slightly higher in females. McGale et al. [23] found higher values for leucine in males than in females (aged 10-70 years). Hagenfeldt et al. [8] investigated the concentrations of amino acids in CSF of adults and found the concentrations of ten amino acids to be higher in males. Females showed a higher concentration for histidine. All these subjects were healthy adult volunteers and age was not taken into account. Ferraro and Hare [3] report sex differences for three amino acids, but not consistent with other authors. Liappis et al. [5] reported higher values for only lysine in boys (age 1-24 months) with no sex differences being found in the group from 2-14 years. In this study 149 children were investigated who were selected using critria similar to our own.

The divergent findings reported in literature can be ascribed to differences in methodology (especially concerning method sensitivity, deproteinization procedure and storage of the samples) and to selection of the control subjects. We have to stress the importance of strict criteria for selecting control groups, acknowledging that collecting a sufficient number of samples is hindered by ethical considerations and practical problems.

We are convinced that our results are a valuable addition to reference values for amino acids in CSF in childhood. Our method also confers the possibility of improving diagnostic capabilities.

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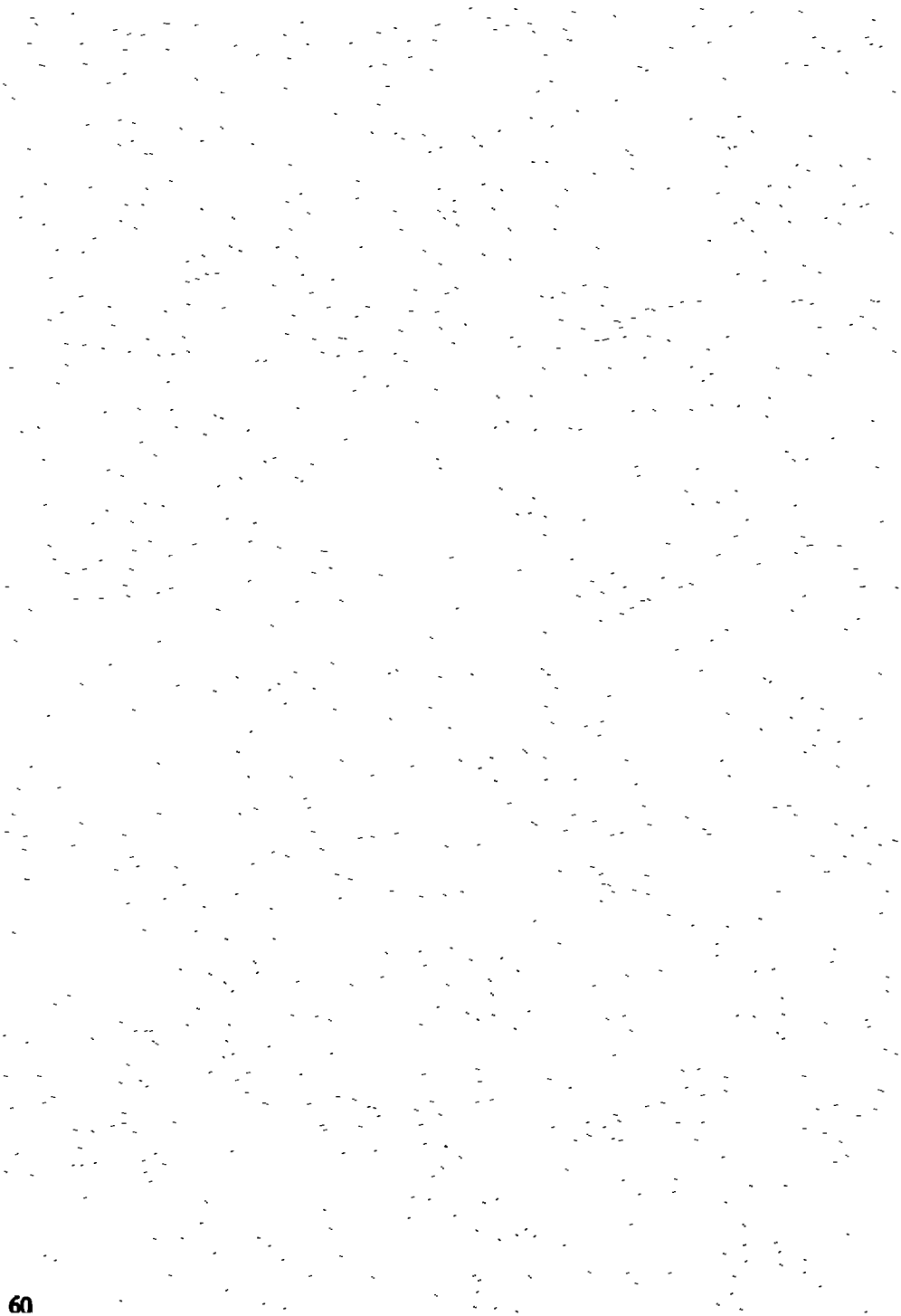

Chapter 4

ARGININOSUCCINIC ACIDURIA: CLINICAL AND BIOCHEMICAL FINDINGS IN THREE CHILDREN WITH THE LATE ONSET FORM, WITH SPECIAL EMPHASIS ON CEREBROSPINAL FLUID FINDINGS OF AMINO ACIDS AND PYRIMIDINES

Chapter 4

Argininosuccinic aciduria: clinical and biochemical findings in three children with the late onset form, with special emphasis on cerebrospinal fluid findings of amino acids and pyrimidines

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Key words: argininosuccinic aciduria, argininosuccinase, urea cycle disorder, cerebrospinal fluid, pyrimidines.

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4.1 Abstract

Three children with the late onset form of argininosuccinic aciduria are presented. The first two are sisters. The clinical features are characterized by mild retardation and ataxia, complicated by episodes of hyperammonemia. All patients showed elevated concentrations of argininosuccinic acid and its anhydrides in all body fluids, most pronounced in cerebrospinal fluid (CSF). Moreover, in Cases 1 and 2, we found elevated concentrations of pseudouridine and uridine limited to CSF, which was not reported before. In Case 3, with some residual activity of argininosuccinate lyase (ASL), we found normal values of these compounds. In urine we found elevated concentrations of uracil in Cases 1 and 2, and orotic acid in Case 2. Plasma showed an elevated concentration of orotic acid in all three patients, uracil was elevated in Case 2, cytidine was elevated in Cases 2 and 3. The results are being discussed and indicate that CSF values of pyrimidines reveal new biochemical abnormalities of brain tissue in urea cycle disorders.

4.2 Introduction

Argininosuccinic aciduria (ASA-uria) is a rare inherited disorder of metabolism, characterized by increased concentration of argininosuccinate and its anhydrides, and citrulline in tissues and body fluids, as well as by mild to marked hyperammonemia. The defect is an absent or decreased activity of argininosuccinase (= argininosuccinic lyase = ASL) (EC 4.3.2.1), the fourth sequential enzyme in the urea cycle. The enzyme is essential for the conversion of argininosuccinic acid into arginine and fumaric acid (fig. 4.1).

The patient population shows a genetic heterogeneity and reveals two variants of ASA-uria: the neonatal type, and the late onset type [4]. In the first variant symptoms including lethargy, poor feeding, tachypnea and seizures may appear within 48 hours after birth, often progressing rapidly to death within a few days. Patients with the second variant show symptoms later on in infancy or childhood. Some patients present with mental retardation and intermittant ataxia. There are also some infants with ASA-uria, which are completely asymptomatic [4].

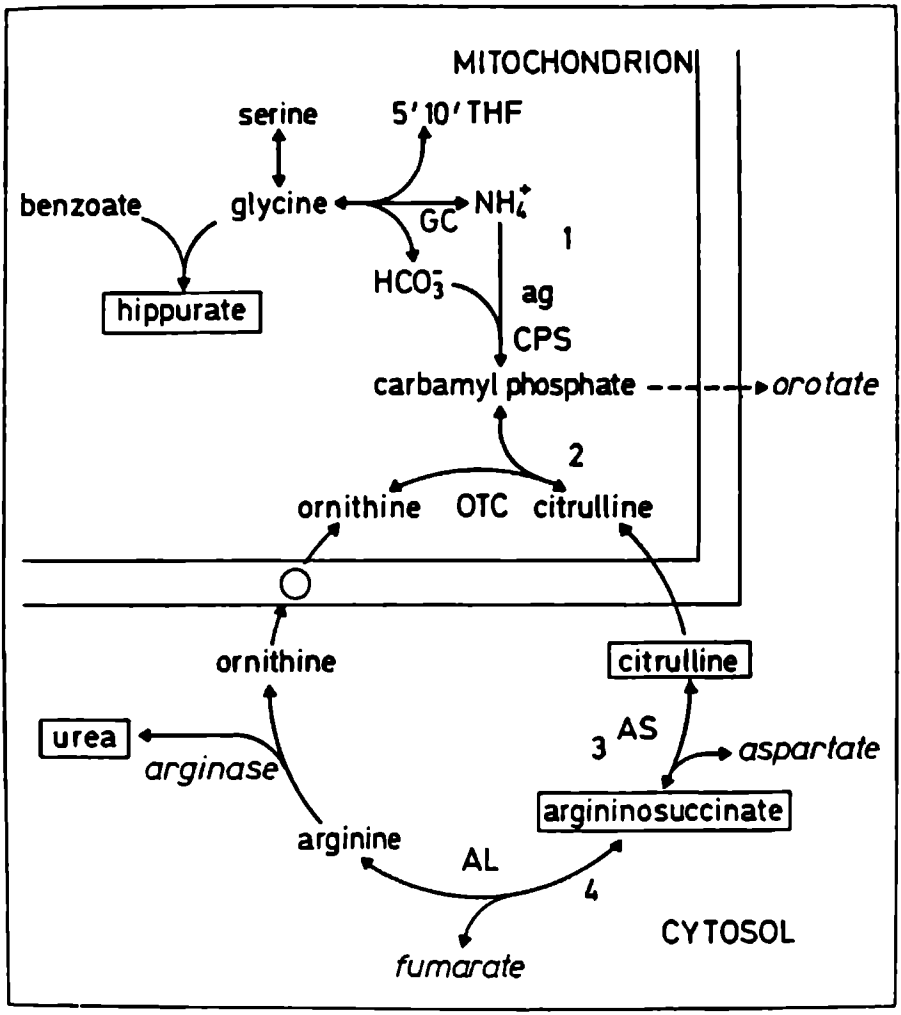

Figure 4.1 *The normal urea cycle. In ASA the cycle is blocked at step 4. The abbreviation ag denotes acetylglutamate (a cofactor for carbamylphosphate synthetase (CPS)); AL, argininosuccinase; AS, argininosuccinate synthetase; OTC, ornithine transcarbamylase; 5'10'THF, 5'10' methylene tetrahydrofolate; and GC, glycine cleavage complex. The excretion products are shown in the boxes. The open circle in the mitochondrial membrane denotes an ornithine transporter. Adapted from Batshaw et al. [3].*

Studies suggest biochemical heterogeneity of the disease [8, 11]. McInnes et al. [9] observed extensive interallelic complementation in ASL deficiency, proving the presence of at least 12 allelic mutations and indicating extensive genetic heterogeneity of ASA-uria [9]. Immunoblot analysis showing a wide variation in the amount and size of ASL cross-reactive material confirms this heterogeneity at protein level [17].

At DNA-level Barbosa et al [2] observed multiple mutations. ASA-uria is one of the few aminoacidopathies in which abnormal cerebral accumulation of amino acids is more pronounced than in serum [15].

New and more sensitive techniques enabled us to study purines and pyrimidines, and amino acids more accurately in CSF [5, 7]. In this paper, we report three cases of the late onset type of ASA-uria, two of them are sisters. Clinical and biochemical features will be discussed with special emphasis on CSF findings of amino acids and pyrimidine contents, in order to get a better insight in the cause and severity of the clinical symptoms reported in this disease.

4.3 Case reports

Case 1

The first child of healthy unrelated parents, was referred to our hospital at an age of 5½ years. Her psychomotor development was slightly delayed, she walked at an age of 19 months. She was clumsy and fell down easily and her hands showed a tremor. There were no behaviour problems. Neurological examination revealed an atactic gait and an intention tremor. Her score at the WISC-R intelligence test was 71. Ammonia was slightly elevated with 66 μmol/l (normal $<$ 50 μmol/l). Laboratory findings revealed the diagnosis of ASA-uria (see Results).

Case 2

A sister of the first patient was subsequently referred at the age of 16 months because of hypotonia and tremor. She was half of a not identical twin. Psychomotor development showed no delay. Neurological examination revealed hypotonia, an intention tremor and atactic hand movements. Laboratory findings revealed an increased ASA excretion. She

received a protein restricted diet (maximum protein intake 2.5 g/kg/day). ASA, however, remained elevated in urine. One year later she was admitted subcomatous after a period of diarrhoea and refusal of feedings. Laboratory findings, revealed a hyperammonemia (120 μmol/l) and grossly elevated concentrations of ASA and its anhydrides. With intravenous rehydration and caloric supplementation she recovered.

Case 3

The child of healthy unrelated parents was already known because of growth retardation (length 10 cm < p10). On the age of 5 years she was admitted for further diagnostic evaluation because of mild psychomotor retardation. Clinical examination showed frontal bossing, a short stature, and no neurological abnormalities. Laboratory findings revealed a normal concentration of ammonia (14.7 μmol/l) and a clearly elevated excretion of ASA and anhydrides. No cause for the growth retardation was detected. Protein reduced diet (maximum protein intake 1.5 g/kg/day) lowered the concentration of ASA, without a beneficial effect on length growth.

4.4 Materials and methods

ASA and its anhydrides as well as other amino acids were determined in urine, plasma and cerebrospinal fluid (CSF). Pyrimidines were determined in urine, plasma and CSF. ASL activity was measured in liver tissue and in fibroblasts.

Amino acid analysis

Amino acid analyses in urine and plasma have been performed on a LKB alpha plus amino acid analyser according to the procedure of the manufacturer. Amino acids in CSF were determined on a Biotronik LC 6001 amino acid analyser using fluorescence detection as described earlier [7].

Pyrimidine analysis

Pyrimidines in urine, plasma and CSF were determined by high performance liquid chromatography according to a previously published method [5] and compared with normal values of purines and pyrimidines in CSF described earlier [6].

Enzyme assays

Enzyme assays were performed according the method of Shih et al. [16]. This assay was validated according to normal laboratory procedures.

4.5 Results

Amino acids in urine, plasma and CSF

In all three body compartments we found elevated concentrations of ASA and its anhydrides in all three patients. ASA and its anhydrides can not be quantified, because they are being interconverted during amino acid analysis. After performing acid hydrolysis, however, we were able to compare the total amount of ASA and anhydrides in CSF and serum. In CSF we found relatively more elevated concentrations of ASA and anhydrides as compared with plasma. We found a CSF-plasma ratio of 2.3 (Case 1). Further results concerning other compounds are presented below per patient.

Case 1 showed in urine a clearly elevated excretion of citrulline (216 μmol/g creatinine, normal: 24-45). In plasma glutamine and citrulline were elevated. In CSF we found elevated concentrations of aspartic acid, citrulline, ethanolamine, tryptophane and histidine.

Case 2, sampled during a period of hyperammonemia, showed in urine only slightly elevated citrulline excretion (59 μmol/g creatinine). In plasma glutamine, alanine and citrulline were elevated. In CSF we found elevated concentrations of serine, asparagine, α-aminoadipic acid, citrulline, methionine, tyrosine, histidine, n-ϵ-methyl-l-lysine and homocarnosine.

Case 3 showed a normal excretion pattern of amino acids in urine. In plasma citrulline was elevated. In CSF we found elevated concentrations of citrulline and homoarginine.

Table 4.1 shows serum levels of amino acids, table 4.2 shows CSF levels of amino acids.

Table 4.1 Concentrations (μmol/l) of some relevant amino acids in serum of three patients with ASA-uria.

Case no.	normal	1	2	3
glutamine	464 - 728	1061 ↑	2986 ↑	526
alanine	150 - 694	251	900 ↑	355
citrulline	7 - 55	333 ↑	96 ↑	62 ↑

Pyrimidines in urine, plasma and CSF

Case 1 showed an uraciluria of 29.7 μmol/mmol creatinine (normal < 8 μmol/mmol creatinine). Other pyrimidines were normal in urine. In plasma orotic acid and uridine were elevated (table 4.3). In CSF of Case 1 we found elevated concentrations of pseudouridine and uridine (table 4.3).

Case 2 showed a clearly increased excretion of uracil (782 μmol/mmol creatinine, normal < 8) and orotic acid (60 μmol/mmol creatinine, normal < 0.02) during a period of hyperammonemia. Other pyrimidines showed normal values in urine. In plasma orotic acid was elevated, cytidine, uridine and uracil were slightly elevated. In CSF of Case 2 we found elevated concentrations of pseudouridine and uridine (table 4.3).

Case 3 had normal excretion of pyrimidines. In plasma orotic acid was elevated, cytidine was slightly elevated. In CSF of Case 3 we found normal concentrations of pyrimidines.

Uracil, cytidine and orotic acid, elevated in urine and plasma, showed normal values in CSF of all three patients (table 4.3).

ASL activity in tissues

In Case 1 ASL activity in liver amounted to 0.001 $\mu mol \cdot min^{-1} \cdot mg^{-1}$protein (normal 0.006-0.020). In Case 3 ASL activity in cultured fibroblasts was 0.006 $\mu mol \cdot min^{-1} \cdot mg^{-1}$protein (normal 0.020-0.027).

Table 4.2 Amino acid concentrations (μmol/l) in CSF of three patients with ASA-uria.

Case no.	normal	1	2	3
taurine	4 - 9	6.9	2.8	7.9
phosphoethanolamine	3 - 7	5.8	4.3	2.3
aspartic acid	2 - 4	4.7 ↑	-	3.0
threonine	14 - 41	23.8	20	29
serine	21 - 44	38	52 ↑	37
asparagine	3 - 8	-	13 ↑	8
α-aminoadipic acid	0 - 1	0.9	1.1 ↑	0.5
glycine	3 - 8	5.6	4.7	4.8
alanine	13 - 31	19.1	27.4	28.1
citrulline	1 - 2	18.6 ↑	10.1 ↑	5.0 ↑
α-aminobutyric acid	1 - 5	2.5	3.3	2.7
valine	9 - 18	11.4	10.7	13.8
methionine	1 - 4	3.8	12.7 ↑	3.8
cystathionine	-	-	-	0.2
isoleucine	3 - 6	2.4	3.2	5.2
leucine	7 - 16	-	7.2	11.1
tyrosine	5 - 13	12.7	16 ↑	9.7
phenylalanine	4 - 14	8.5	13.9	8.9
γ-aminobutyric acid	0 - 1	0.9	< 0.02	0.8
ethanolamine	9 - 26	26.8 ↑	20.1	13.3
hydroxylysine	-	0.2	0.2	0.02
tryptophane	1 - 4	6.1 ↑	1.4	3.5
ornithine	2 - 6	3.9	1.1	3.9
lysine	10 - 28	16.9	10.7	18.7
histidine	9 - 19	20.1 ↑	44.6 ↑	12.3
n-ε-methyl-l-lysine	0 - 7	6.7	7.9 ↑	4.2
3-methyl-histidine	0 - 1	0.6	0.5	0.2
homocarnosine	2 - 11	10.4	16.6 ↑	5.1
arginine	13 - 31	11.3	6.6	21.0
homoarginine	-	-	-	0.7 ↑

Table 4.3 *Purines and pyrimidines in CSF and plasma of patients with ASA, values in μmol/l.*

Case no.	normal	1	2	3
CSF values:				
pseudouridine	< 0.02	1.00 ↑	0.68 ↑	< 0.02
cytidine	0 - 0.72	0.28	0.28	< 0.02
uridine	0.96 - 3.28	3.43 ↑	6.96 ↑	2.13
inosine	0.14 - 0.86	< 0.02	0.69	0.70
orotic acid	< 0.02	< 0.02	< 0.02	< 0.02
uracil	0 - 2.62	< 0.02	< 0.02	< 0.02
plasma values:				
pseudouridine	0.5 - 26.2	7.43	8.64	4.25
orotic acid	< 0.02	6.10 ↑	3.22 ↑	3.25 ↑
cytidine	< 2	1.67	2.12 ↑	2.39 ↑
uridine	0.5 - 5	4.48	5.33 ↑	1.61
inosine	< 1.5	0.43	0.49	0.43
uracil	< 2	1.05	2.03 ↑	0.03

4.6 Discussion

The clinical features of our patients are characterized by the presentation of mild symptoms during childhood. The only symptoms are mild retardation and ataxia. Case 3 showed a growth retardation, but it is unlikely that this can be ascribed to the ASA-uria. Cases 1 and 3 were discovered during a routine screening because of a mild retardation. They never suffered from episodes of hyperammonemia and subsequent clinical signs of metabolic dysregulation, which are characteristic for this inherited metabolic disorder. Case 2 was suspected to be suffering from ASA-uria because of a positive family history. She was the only one suffering an episode of hyperammonemia with clinical symptoms of a metabolic dysregulation. The variability of ASA-uria, in age of onset, severity, and degree of residual enzyme activity is large [4]. This explains the absence of clinical symptoms in Case 3, having a residual enzyme activity in fibroblasts. In this patient the citrulline concentration in blood and CSF was only mildly elevated.

Hyperammonia results in increased de novo synthesis of pyrimidines. The increased carbamylphoshate, synthesized in mitochondria, cannot be fully utilized by the urea cycle. Hence, the excess carbamylphosphate leaks into the cytosol where it serves as a source for pyrimidine biosynthesis, leading to an increased flux for pyrimidine synthesis in the cytosol.

We found an increased excretion of uracil in Cases 1 and 2, and orotic acid in Case 2. This can be an expression of increased plasma level. In ASA-uria Naylor and Cederbaum [10] mentioned the presence of orotic aciduria and reported it as an unpublished observation. Van Gennip et al. [21] reported slightly elevated urinary levels of orotic acid. In the latter report uracil, uridine and pseudouridine were within the normal range in patients' urine.

To our knowledge, CSF contents of pyrimidines have not been reported before in an urea cycle defect. We were particularly interested in the CSF concentrations of pyrimidines, because the urea cycle is present in brain [19]. Abnormalities in CSF may more accurately reflect the diversity of the clinical symptoms. In ASA-uria the concentration of ASA and its anhydrides in CSF is found to be twice to four times that noted in plasma [15]. We found a ratio of 2.3.

The most striking result in our study is an elevated concentration of pseudouridine in CSF in Cases 1 and 2, the patients with the lowest ASL activities. In Case 3, having a substantial residual activity we found no pseudouridine. Normally pseudouridine is not detectable in CSF. Pseudouridine is a t-RNA catabolyte and its excretion in urine is related to growth rate or cell turnover and can be associated with tissue destruction [14]. In chronic diseases, in which cell turnover is assumed to be higher than normal, pseudouridine excretion is increased [12]. Moreover, in both patients a moderately elevated concentration of uridine was demonstrated in CSF.

Phosphoribosylpyrophosphate (PRPP) is a substrate for purine as well as pyrimidine de novo synthesis. Thus, increased pyrimidine de novo synthesis results in a shift of PRPP available away from the purine pathway and can conceivably result in decreased purine biosynthesis. In rat liver high levels of ammonia clearly stimulated pyrimidine de novo synthesis and inhibited purine de novo synthesis. In rat brain, UMP synthesis by the de novo pathway was also stimulated by high ammonia, although purine de novo sythesis remained constant. As the liver provides a major portion of purine nucleotides for the

brain, a decrease in purine biosynthesis in liver could influence brain nucleic acid synthesis.

A decreased rate of purine nucleotide biosynthesis could at least partly explain the retardation of growth seen in infants and children with chronic hyperammonia [18]. The pyrimidine de novo pathway is the predominant route in pyrimidine nucleotide biosynthesis [20]. However, two pyrimidine biosynthetic pathways exist, the de novo and the salvage pathway, which utilizes exogenous nucleosides. Uridine kinase is a major enzyme of the salvage pathway. Uridine is converted into UMP by this enzyme. The conversion is ATP dependent. As mentioned, hyperammonia can result in increased pyrimidine de novo biosynthesis, e.g. increased UMP, and decreased purine biosynthesis, e.g. depleted ATP. Both, elevated UMP and depleted ATP, may account for a lower conversion of uridine into UMP by uridine kinase. This may explain the elevated uridine levels observed in CSF. Uridine has been described to have anticonvulsant effects in animals with experimental seizure phenomena, indicating that this compound may play a role of importance in regulating nervous system activity [13].

Elevations of uracil and orotic acid, demonstrated in urine (uracil in Cases 1 and 2, and orotic acid in Case 2) and plasma (uracil in Case 2 and orotic acid in all three patients), were not present in CSF of all three patients. Regarding the uridine elevation in CSF, it puzzles us why this is not resulting in an uracil elevation in CSF. The presence of isoenzymes of uridine kinase offers an explanation. Four types of uridine kinase are known [1], and one form is present in rat brain.

It can be concluded that CSF values of pyrimidines in ASA-uria and probably other urea cycle disorders reveal new biochemical abnormalities of the brain tissue.

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Chapter 5

DISTURBANCES OF CEREBRAL PURINE AND PYRIMIDINE METABOLISM IN YOUNG CHILDREN WITH CHRONIC RENAL FAILURE

Chapter 5

Disturbances of cerebral purine and pyrimidine metabolism in young children with chronic renal failure

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5.1 Abstract

Chronic renal failure during childhood may be associated with delayed cognitive development. From 10 children with chronic renal failure, aged 2-59 months, plasma and cerebrospinal fluid (CSF) purines and pyrimidines have been determined. A marked increase of pseudouridine and cytidine was demonstrated in CSF of 10 and 8 children, respectively. The plasma concentration of pseudouridine was increased in a varying degree to a maximal value of more than 10 times the upper limit of normal. The plasma concentration of cytidine showed only moderately elevated values. In 3 children the study of CSF and plasma was repeated 6 weeks after the start of continuous ambulatory peritoneal dialysis. The abnormal concentrations of pseudouridine and cytidine were still present in CSF and plasma. Further studies are necessary to elucidate the cause of this unknown biochemical aberration of the central nervous system.

5.2 Introduction

Chronic renal failure in infancy and childhood can be associated with delayed cognitive development. This delay in development remains as yet unexplained. Extensive studies in adults fail to explain adequately the effects of uremia on the central nervous system [1-3]. A high incidence of neurodevelopmental disturbances in uremic children have been reported by several authors [4, 5]. To evaluate uremic encephalopathy many approaches have been applied. Neuro-electrophysiological, psychological and biochemical studies in cerebrospinal fluid (CSF) have been performed. In the latter, amino acid derangements and imbalance of neurotransmitters are the most striking features [1]. It is, however, unknown if and to what extend these derangements give rise to the clinical features of uremic encephalopathy. Studies explaining the pathogenetic role of chronic renal failure in neurodevelopmental dysfunction in childhood are still lacking. In the only study related to purine and pyrimidine metabolism an increased concentration of pseudouridine was found in serum of adult patients undergoing continuous ambulatory peritoneal dialysis (CAPD) [6]. We investigated the content of purines and pyrimidines in CSF and plasma of 10 children with chronic renal failure. The concentration in CSF is a better reflection of

cerebral metabolism than the plasma concentration, as the concentration in CSF is in equilibrium with the brain interstitial fluid.

5.3 Materials and methods

From 10 children with chronic renal failure, aged 2-59 months, plasma and CSF samples were collected for determining the content of purines and pyrimidines. This study was approved by the local ethical comittee. Some clinical features of these patients are summarized in table 5.1. Three of them (nrs. 2, 5 and 8) were treated by CAPD a few months later. From these patients samples of plasma and CSF were collected a second time after CAPD therapy for at least six weeks, in order to study the possible effect of CAPD on the content of purines and pyrimidines.

Table 5.1 *Some clinical and laboratory features of the patients.*

patient no.	age months	serum creatinin μmol/l	clearance of creatinin ml/min/1.73 m^2	underlying disease
1	2	83	24.0	renal dysplasia
2	2	275	7.5	urethral valves
3	7	384	6.6	renal dysplasia
4	8	75	32.9	renal dysplasia
5	8	286	9.9	urethral valves
6	8	137	19.8	renal dysplasia
7	8	204	12.3	renal dysplasia
8	26	500	7.2	urethral valves
9	32	869	3.7	glomerulosclerosis
10	59	485	8.0	glomerulosclerosis

Purines and pyrimidines were determined by high-performance liquid chromatography according to a previously published method [7] and compared with normal values of purines and pyrimidines in CSF described before [8] and with control children for plasma. Heparinized plasma was centrifuged immediately after venipuncture.

Table 5.2 Concentrations (μmol/l) of pseudouridine (normal ≤ 0.02) and cytidine (normal ≤ 1.07) in CSF of patients with chronic renal failure before (n = 10) and after (n = 3) start of CAPD.

Patient no.	age, months		Pseudouridine		Cytidine	
	before	after CAPD	before	after CAPD	before	after CAPD
1	2		12.7		11.2	
2	2	6	37.8	36.4	14.7	13.3
3	7		17.3		4.2	
4	8		9.8		10.8	
5	8	19	12.0	21.5	5.1	11.8
6	8		21.4		10.0	
7	8		6.8		9.3	
8	26	28	28.8	14.2	13.6	8.7
9	32		25.9		0.50	
10	59		10.9		0.47	

5.4 Results

A typical chromatogram of CSF from a patient with chronic renal failure is shown in fig. 5.1. For comparison, a chromatogram of a control patient is shown in fig. 5.2.

In all children a marked increase of pseudouridine was found in CSF with values ranging from 6.8-37.8 μmol/l (normal < 0.02 μmol/l) (table 5.2). In plasma collected at the same time as CSF elevated values for pseudouridine were observed in 6 out of 7 patients, with values ranging from 34.6-355 μmol/l (normal 0.5-26.2) (table 5.3).

Table 5.3 *Plasma values of purines and pyrimidines in patients with chronic renal failure (n = 7, range in μmol/l).*

	control children	patients						
		1	2	5	7	8	9	10
uracil	< 2	3.1	2.2	4.0	0.85	3.3	< 0.10	0.47
pseudouridine	0.5-26.2	25.5	73.4	40.5	34.6	45.8	255	355
uric acid	120-400	522	648	873	431	556	446	232
cytidine	< 2	2.6	1.4	3.1	2.9	2.9	5.4	2.2
hypoxanthine	< 4	15.8	21.6	15.9	10.8	6.6	20.4	6.8
xanthine	< 2	3.8	4.9	3.5	2.4	2.8	6.9	2.6
uridine	0.5-5	6.7	3.8	3.1	4.1	6.4		3.2
inosine	< 1.5	1.45	0.80	0.94	0.63	0.90	2.24	3.68

In 8 out of 10 children we also found a marked increase of cytidine in CSF with values ranging from 4.2-14.7 μmol/l (normal value ≤ 1.07 μmol/l). Plasma also showed moderately elevated values for cytidine in 6 out of 7 patients, with values ranging from 2.2-5.4 μmol/l (normal < 2 μmol/l). The increase of the concentration of pseudouridine and cytidine in CSF in 3 patients remained present after CAPD treatment was instituted for a period of at least 6 weeks (table 5.2).

Concerning the other purine and pyrimidine compounds measured in CSF, the following results were obtained (table 5.4); uridine showed in 3 out of 10 cases slightly decreased values in CSF and in patient nr. 4 a substantial decrease. Increased values to a varying degree were observed in CSF in 3 cases for hypoxanthine, in 5 cases for xanthine and in 5 cases for inosine. Except for patient nr. 10, in whom both xanthine and inosine were increased, all other patients (with the exception of nr. 5) showed an increase in either one of both compounds. Uracil, uric acid, guanosine, adenosine, adenine and thymine showed normal values in CSF.

Table 5.4 Concentrations (μmol/l) of other purines and pyrimidines in CSF of patients with chronic renal failure.

patient no.	Hypoxanthine (0.64-4.12)	Xanthine (0-4.16)	Uridine (0.33-3.71)	Inosine (0.15-0.59)
1	3.95	7.25	0.37	0.40
2	3.78	5.62	0.20	0.54
3	1.97	2.35	1.52	0.69
4	3.45	6.55	0.12	0.25
5	1.45	1.88	0.29	0.54
6	4.74	1.74	0.72	0.87
7	2.55	1.55	0.52	0.69
8	3.61	6.08	0.29	0.40
9	6.62	3.74	2.19	1.33
10	5.05	4.23	1.86	0.97

Values in parentheses indicate normal range.

Plasma values of the mentioned compounds were normal in the 7 patients measured, except for uric acid, hypoxanthine and xanthine which showed elevated levels in plasma (table 5.3). Four out of 7 patients showed an elevated level of uracil in plasma. Two patients showed an elevated level of uridine, 2 patients showed an elevated level of inosine.

Moreover, we found in the chromatograms of CSF of all patients a large still unknown compound (fig. 5.1). This unknown compound was also present in plasma.

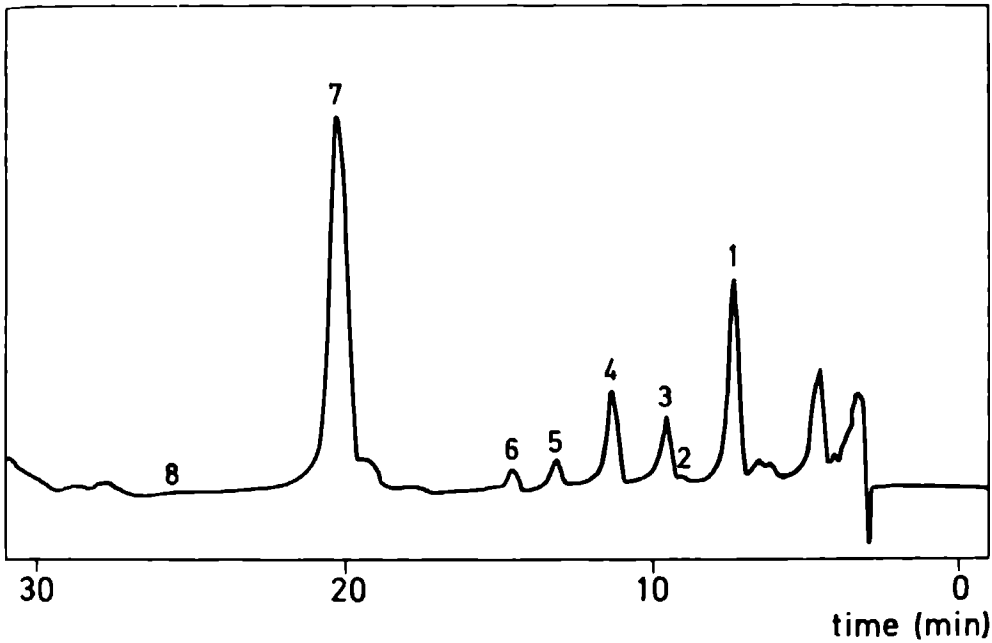

Figure 5.1 *A typical chromatogram of CSF from a patient with chronic renal failure. Peak identification: 1 = pseudouridine; 2 = uric acid; 3 = cytidine; 4 = hypoxanthine; 5 = xanthine; 6 = uridine; 7 = unknown; 8 = inosine.*

5.5 Discussion

The most outstanding result in our study of CSF was the strong increase of the concentration of pseudouridine in all patients and of cytidine in 8 out of 10 patients. Pseudouridine, a t-RNA catabolite, is associated with tissue destruction. Pseudouridine excretion in urine is also more generally related to growth rate or cell turnover and was thought to be a possible tumor marker [9]. In chronic diseases, in which cell turnover is assumed to be higher than normal, pseudouridine excretion is increased [10].

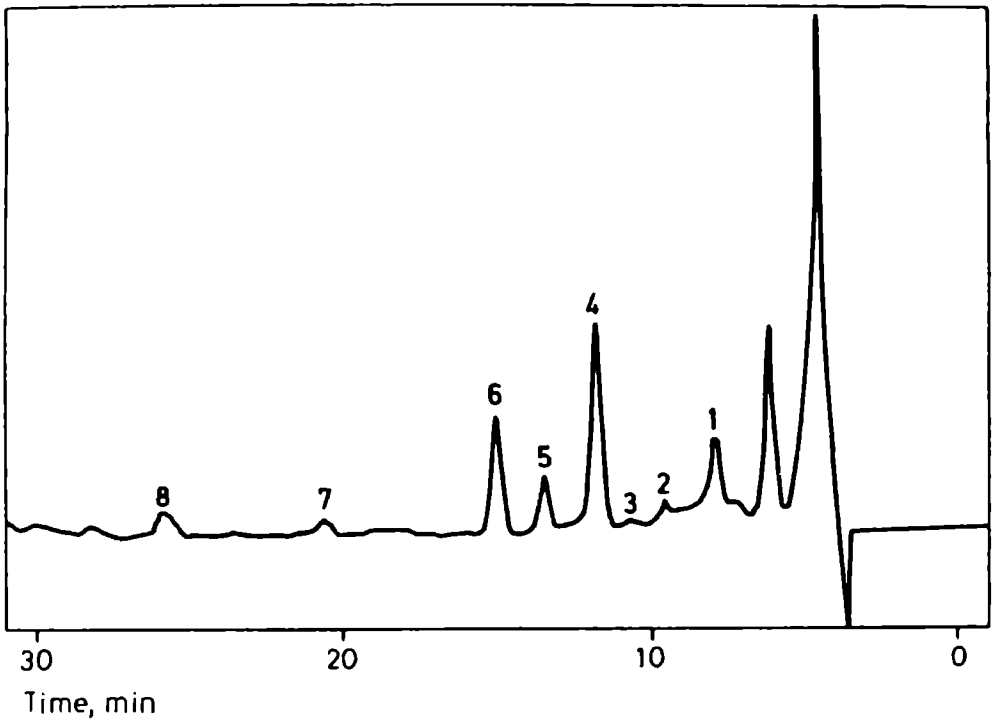

Figure 5.2 *A normal chromatogram of CSF of a healthy child.*
Peak identification: 1 = uracil; 2 = uric acid; 3 = cytidine; 4 = hypoxanthine; 5 = xanthine; 6 = uridine; 7 = unknown peak; 8 = inosine.

Pseudouridine excretion gradually decreases with age, with a rapid fall in the first year of live [11, 12]. Schoots et al. [6] and Schoots [13] demonstrated high pseudouridine concentrations in serum from CAPD patients but not in serum of patients treated with hemodialysis.

They suggested two possible explanations for this phenomenon: nonclinical peritonitis accompanied by cell death or increased protein synthesis in CAPD patients. Both these conditions are associated with a higher t-RNA turnover. Our observations leave no role for CAPD in the elevation of pseudouridine because this increase remains elevated.

Secondary effects associated with the derangement state of uremia formed their second speculation. The enormous increase of pseudouridine in CSF (factor 1,000) compared to the increase in plasma (factor 10) suggests a cerebral abnormality in our patients.
The much greater increase of cytidine in CSF, resulting in a much higher concentration in CSF compared to plasma, indicates that the metabolic disturbance related to cytidine is more pronounced in cerebral tissue. The high cytidine elevation in CSF of uremic patients resulting in a concentration surpassing the plasma concentration points to a hitherto unknown biochemical disturbance of the central nervous system. We cannot explain why cytidine is not elevated in the 2 oldest children. It is possible that maturation effects would explain this discrepancy, which would mean that younger children show greater derangements, or less probably it could be related to the diagnosis of dysplasia. In patients with severely disturbed renal function and urethral valves renal dysplasia is a common feature. A secondary inhibition of a metabolic pathway specific to cerebral tissue might explain the increase of cytidine. Inhibition of an isoenzyme involved in the metabolism of cytidine and located mainly in the cerebrum could offer such an explanation [14]. Four types of enzymes of uridine-cytidine kinase (EC 2.7.1.48) are known [15]. Type four is the form present in rat brain and is a tetramer of the first form. This enzyme could be more susceptible to the influence of abnormalities observed in uremia such as increase of phenolic acid and aromatic or alifatic amines or intracellular calcium content [1]. It is remarkable, however, that uridine is normal or slightly decreased in CSF, suggesting an influence on cytidine-deaminase (EC 3.5.4.5) activity. It is known that cytidine nucleotides are involved as cofactors in phospholipid metabolism in brain [16]. In brain, cytidine is converted in vivo into CMP, and by a stepwise mechanism to CDP, CTP (fig. 5.3), and finally to ethanolamine and choline cytidine nucleotides [17]: CDP-ethanolamine is the limiting factor for cephalin synthesis and CDP-choline is the limiting factor for lecithin sythesis [16]. We wonder whether the disturbance of pyrimidine metabolism could result in an impaired cognitive development.
Our results indicate that, if the pyrimidine disturbances are involved in the impaired cognitive development, CAPD will not influence this cognitive development.
Concerning purine metabolism, we observed constant elevated plasma levels of xanthine, hypoxanthine and uric acid (table 5.3). It is well known that uric acid is elevated in serum

in impaired renal function. An increase in plasma levels of hypoxanthine and xanthine can be expected when the renal function is disturbed.

Normally these substances are regulated by glomerular filtration and tubular reabsorption [18]. The constant elevation of these compounds, however, was not reflected by a constant increase in CSF. Uric acid was even normal in CSF. These findings are consistent with the very high and very low concentration of hypoxanthine-guanine phosphoribosyltransferase and xanthine oxidase, respectively, in brain parenchyma [19].

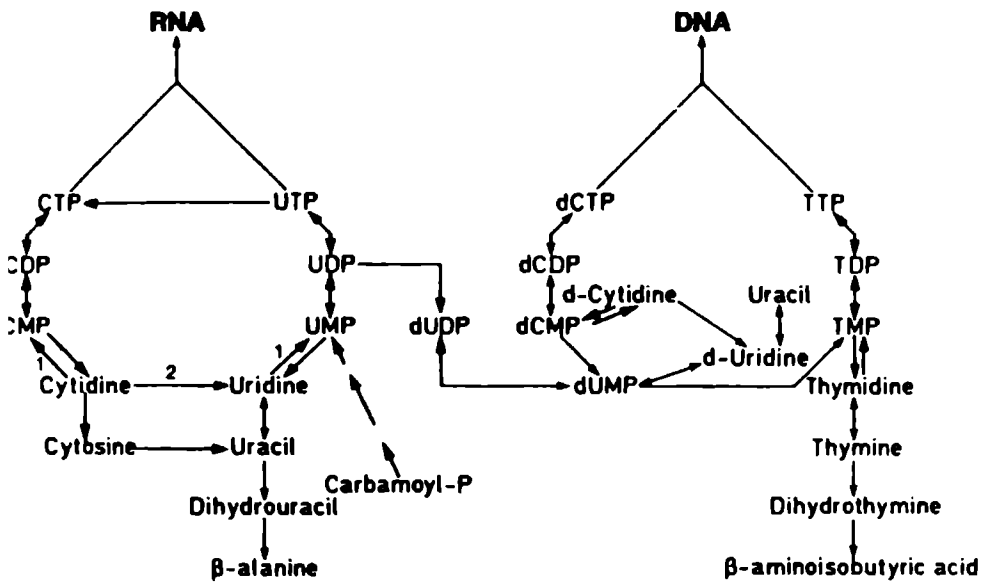

Figure 5.3 *A schematic representation of pyrimidine metabolism.*
1 = Uridine-cytidine kinase; 2 = Cytidine deaminase.

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Chapter 6

CEREBROSPINAL FLUID LEVELS OF AMINO ACIDS IN INFANTS AND YOUNG CHILDREN WITH CHRONIC RENAL FAILURE

Chapter 6

Cerebrospinal fluid levels of amino acids in infants and young children with chronic renal failure

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6.1 Abstract

Chronic renal failure (CRF) is associated, especially in young children, with delayed cognitive development of unknown origin. As cerebrospinal fluid (CSF) reflects the composition of the extracellular fluid of the brain, not only plasma but also CSF amino acids concentrations were determined in 8 infants (age 2-8 months) and 3 children (age 26, 32 and 56 months) with CRF (creatinine clearance 13 $\pm$ 9 ml/min/1.73m^2). In three of these children investigations were repeated after six weeks of CAPD treatment.

In the infants, a significant decrease was found in CSF of α-aminobutyric acid, valine, isoleucine, leucine, tyrosine, tryptophane, histidine and n-ϵ-methyl-l-lysine, whereas there was a significant increase of 3-methylhistidine. In plasma serine, valine, leucine, tyrosine, and histidine were significantly decreased, whereas there was a significant increase of aspartic acid, citrulline, and 3-methylhistidine.

These abnormalities remained constant after the start of CAPD except for the normalisation in CSF and plasma of 3-methylhistidine.

These data indicate a generalized disturbance of amino acids in young children with CRF. An abnormal substrate is offered to the neurons and astroglia in children with CRF.

6.2 Introduction

Chronic renal failure (CRF) can be associated with delayed cognitive development in children [1-4], the exact cause of this delay being still unknown [5]. It has been assumed that infants are more susceptible to develop an uraemic encephalopathy, because of the significant growth and maturation of the brain that occurs during the first years of life [4]. Besides electrophysiological and psychological aspects, several investigators examined the relationship between amino acid concentrations in urine, plasma and muscle cells in chronic renal disease [6-8]. Cerebrospinal fluid (CSF) studies - the clinician's access to the brain - however have only been performed in adults [2, 5, 9, 10]. We already carried out CSF studies of purines and pyrimidines in children with CRF [11]. A new, more sensitive technique enabled us to study amino acids in CSF more accurately [12]. Not the

plasma amino acid concentration but the CSF amino acid concentration reflects the extracellular amino acid concentration of the brain interstitial fluid.
In order to contribute to the understanding of the developmental delay of children with CRF, not only plasma but also CSF amino acid concentrations were determined in 11 children with chronic renal failure. Three of these children were treated by continuous ambulatory peritoneal dialysis (CAPD) several months later. Amino acid concentrations in CSF were repeated after at least six weeks of therapy.

6.3 Materials and methods

From 11 children with CRF, aged 2-59 months, plasma and CSF samples were collected for determining the content of amino acids. After collection by venapuncture, 1 ml of heparinized plasma was put aside for the present investigation. The CSF samples were kept frozen at -70° C until analysis. Just before analysis the samples were thawed and deproteinized according to previous published methods [12].
The study was approved by the local ethical committee.
Some clinical features of the patients are summarized in table 6.1. Three of them (nrs. 2, 6 and 9) were treated by CAPD a few months later. In these patients samples of plasma and CSF were collected a second time after CAPD therapy for at least six weeks. Amino acids were determined according to a previously published method [12] and compared with normal values of amino acids in CSF described before [12] and with plasma values of 26 age-matched control children.

6.4 Statistics

Amino acids in plasma and CSF displayed log-normal distributions. So the comparisons with normal values were made on the log-transformed data, using Student's t-test. The significance level α is set as 0.01.

Table 6.1 *Clininal and laboratory features of the patients.*

patient no.	age months	serum creatinin μmol/l	clearance of creatinin ml/min/1.73 m^2	underlying disease
1	2	83	24.0	renal dysplasia
2	2	275	7.5	urethral valves
3	4	322	7.3	urethral valves
4	7	384	6.6	renal dysplasia
5	8	75	32.9	renal dysplasia
6	8	286	9.9	urethral valves
7	8	137	19.8	renal dysplasia
8	8	204	12.3	renal dysplasia
9	26	500	7.2	urethral valves
10	32	869	3.7	focal glomerulo-sclerosis
11	59	485	8.0	focal glomerulo-sclerosis

6.5 Results

Plasma amino acids

In seven patients (nr. 1, 2, 6, 8, 9, 10 and 11) we were able to determine amino acids in plasma obtained simultaneously with the CSF. In the four youngest children the plasma values were compared with age-matched controls. Significantly decreased values in plasma were obtained in the four infants for serine, valine, leucine, tyrosine, and histidine (table 6.2).

Significantly increased values in plasma were observed for aspartic acid, citrulline, 3-methylhistidine (table 6.2).

In the three older children (nrs. 9, 10, and 11) compared with age-matched controls, elevated concentrations were found in plasma for glutamine, glycine, citrulline, and 3-methylhistidine.

Table 6.2 Amino acids in plasma (μmol/l) of 4 children (nrs. 1, 2, 6 and 8) with renal failure

	Reference values (n = 26)			4 children with renal failure				
	$P_{2.5}$	P_{50}	$P_{97.5}$	Nrs. 1	2	6	8	p-value*)
taurine	38	76	152	138	99	20	61	0.44
aspartic acid	4	11	32	56	32	12	33	< 0.001 ↑
threonine	92	133	193	195	212	63	135	0.83
serine	98	150	232	104	137	52	97	< 0.001 ↓
asparagine	36	48	64	46	38	27	50	0.042
glutamic acid	48	86	156	159	185	58	83	0.19
glutamine	500	712	1013	353	660	203	892	0.27
glycine	117	198	337	231	464	106	313	0.82
alanine	237	338	480	342	344	196	392	0.40
citrulline	11	20	35	43	35	28	68	< 0.001 ↑
α-aminobutyric acid	6	13	29	78	7.4	0.0	12	0.50
valine	136	194	276	103	166	144	111	< 0.001 ↓
cystine	5	26	149	14	14	4.2	0	0.21
methionine	15	24	40	35	25	44	25	0.054
isoleucine	49	75	115	47	59	61	59	0.021
leucine	82	127	195	71	88	91	70	< 0.001 ↓
tyrosine	38	64	108	35	42	48	43	0.003 ↓
phenylalanine	32	49	76	56	59	49	46	0.61
ornithine	50	92	168	78	68	35	79	0.041
lysine	114	163	231	155	156	96	158	0.12
histidine	83	102	124	63	79	49	76	< 0.001 ↓
1-methyl-histidine	-	-	-	.	6.2	6.2	0.0	-
3-methyl-histidine	-	-	-	25	4.4	6.3	48	↑
arginine	44	72	117	80	98	34	82	0.71

*) Student's t-test (logtransformed data)

Table 6.3 CSF amino acids (μmol/l) of 8 children (nrs. 1 - 8) with renal failure

	Reference values			8 children with renal failure			
	$P_{2.5}$	P_{50}	$P_{97.5}$	Min	P_{50}	Max	p-value*)
taurine	5.32	8.52	13.65	3.37	6.21	9.09	0.043
phosphoethanolamine	3.05	4.53	6.74	2.57	3.61	5.03	0.042
aspartic acid	3.70	6.24	10.52	3.22	4.54	6.57	0.016
threonine	15.09	44.32	130.17	19.44	29.26	62.20	0.22
serine	29.57	30.39	85.89	24.68	32.22	45.81	0.72
asparagine	4.46	8.56	16.42	4.95	5.92	7.88	0.013
α-aminoadipic acid	0.23	0.52	1.18	0.17	0.39	0.53	0.048
glycine	3.82	5.52	7.98	4.13	5.05	8.79	0.99
alanine	16.49	26.00	40.98	16.20	30.91	37.12	0.64
citrulline	1.25	3.12	7.80	2.65	3.37	4.10	0.77
α-aminobutyric acid	1.95	2.88	4.25	0.31	1.09	1.88	< 0.001↓
valine	11.91	18.70	29.37	6.62	9.37	16.71	< 0.001↓
methionine	1.55	3.40	7.44	2.09	3.06	7.00	0.98
isoleucine	4.66	7.38	11.71	3.99	4.71	7.46	0.003↓
leucine	12.39	15.58	19.59	5.14	8.17	13.05	< 0.001↓
tyrosine	6.86	13.01	24.67	4.15	5.39	11.48	< 0.001↓
phenylalanine	5.15	10.77	22.50	6.42	7.35	10.02	0.023
γ-aminobutyric acid	0.00	0.05	0.13	0.01	0.04	0.28	0.69
tryptophane	2.10	3.96	7.47	1.91	2.48	3.53	0.007↓
ornithine	2.84	7.38	19.17	3.54	4.96	8.06	0.13
lysine	11.61	20.57	36.47	10.28	14.69	19.98	0.017
histidine	10.46	17.75	30.12	6.78	9.51	14.59	0.001↓
n-ε-methyl-l-lysine	1.64	3.12	5.94	0.77	1.61	3.52	0.002↓
3-methyl-histidine	0.14	0.42	1.21	0.87	2.05	2.61	< 0.001↑
homocarnosine	5.54	8.08	11.79	4.64	9.41	13.89	0.41
arginine	11.92	19.44	31.70	12.57	19.37	27.11	0.89

*) Student's t-test (logtransformed data)

Table 6.4 CSF amino acids (μmol/l) of 3 children (nrs. 2, 6 and 9) with renal failure, before and after CAPD

	Reference values			3 children with renal failure					
	$P_{2.5}$	P_{50}	$P_{97.5}$	before CAPD	after CAPD	before CAPD	after CAPD	before CAPD	after CAPD
taurine	5.32	8.52	13.65	8.22	6.73	3.37	3.67	4.08	3.11
phosphoethanolamine	3.05	4.53	6.74	4.25	4.83	2.86	.	3.00	2.92
aspartic acid	3.70	6.24	10.52	3.54	3.11	4.91	3.40	2.58	2.50
threonine	15.09	44.32	130.17	24.45	17.50	50.99	19.96	9.36	14.81
serine	29.57	30.39	85.89	25.49	26.19	24.68	24.55	22.00	29.06
asparagine	4.46	8.56	16.42	5.75	5.45	5.67	3.32	3.02	3.57
α-aminoadipic acid	0.23	0.52	1.18	0.38	1.00	0.17	0.10	0.11	0.17
glycine	3.82	5.52	7.98	4.79	6.49	4.13	3.78	6.12	5.39
alanine	16.49	26.00	40.98	31.35	36.82	16.20	28.11	16.30	20.71
citrulline	1.25	3.12	7.80	3.66	3.51	3.11	2.72	2.85	2.02
α-aminobutyric acid	1.95	2.88	4.25	0.77	0.49	0.31	0.63	0.47	0.63
valine	11.91	18.70	29.37	8.65	6.99	9.45	8.68	.	8.66
methionine	1.55	3.40	7.44	2.61	1.98	3.01	1.88	2.14	1.93
isoleucine	4.66	7.38	11.71	4.13	5.77	5.84	3.66	4.28	3.95
leucine	12.39	15.58	19.59	7.17	4.49	11.95	7.82	6.09	5.35
tyrosine	6.86	13.01	24.67	4.62	2.90	5.03	3.41	3.68	9.64
phenylalanine	5.15	10.77	22.50	7.09	6.41	7.56	6.88	6.03	7.77
γ-aminobutyric acid	0.00	0.05	0.13	0.04	0.00	0.00	0.00	0.10	0.13
tryptophane	2.10	3.96	7.47	2.48	1.42	.	.	0.95	0.87
ornithine	2.84	7.38	19.17	4.63	3.45	3.93	3.04	12.60	12.23
lysine	11.61	20.57	36.47	19.03	13.06	13.32	13.63	9.01	7.25
histidine	10.46	17.75	30.12	8.21	7.80	6.78	8.44	1.21	0.64
n-ε-methyl-l-lysine	1.64	3.12	5.94	1.15	0.00	3.52	1.51	0.95	0.87
3-methyl-histidine	0.14	0.42	1.21	2.49	1.49	2.41	0.86	3.68	1.15
homocarnosine	5.54	8.08	11.79	10.24	8.21	4.64	5.98	4.74	4.53
arginine	11.92	19.44	31.70	25.10	27.24	12.57	11.54	16.01	19.00

In three patients (nrs. 2, 6, and 9) plasma amino acid concentrations were repeated after treatment with CAPD.
Valine, leucine and histidine improved in two patients.
In all three aspartic acid became normal. In two patients 3-methylhistidine showed lower, but still increased values. Glycine showed even higher values in two patients.

CSF amino acids

In CSF of the 8 infants (nrs. 1-8) we found a significant decrease of: α-aminobutyric acid, valine, isoleucine, leucine, tyrosine, tryptophane, histidine, and n-ϵ-methyl-l-lysine (table 6.3).
A significant increase of 3-methylhistidine ($p < 0.001$, table 6.3) was measured.
In the three older children (nrs. 9, 10, and 11) we found also decreased values for α-aminobutyric acid. Valine, leucine, tyrosine, tryptophane, lysine, histidine and n-ϵ-methyl-l-lysine were decreased in either one or two patients. 3-methylhistidine was increased in all three older children. Alanine, citrulline and ornithine showed increases in two patients.

In three patients (nrs. 2, 6, and 9) the amino acid concentrations in CSF were determined again after more than six weeks of CAPD-treatment. No normalisation of the pre-CAPD values was observed except for 3-methylhistidine, the only compound with a significant increase (table 6.4).

Valine, leucine, tyrosine, and histidine concentrations were decreased both in plasma and CSF. 3-Methylhistidine was the only compound increased in both compartments. For the other compounds the deviations in either plasma or CSF can not be related.

6.6 Discussion

Before discussing our results in children with CRF, the known factors which are determining the amino acid concentration in CSF will be briefly reviewed. The amino acid concentration is known to be much lower in plasma compared to CSF except for

glutamine. CSF is formed by the choroid plexus, bathes the brain and is drained via the arachnoid villi (fig. 6.1). The brain compartment is in close contact with the blood at the blood-brain barrier (BBB), located at the brain capillary endothelium sealed with tight junctions, and via the CSF at the blood-CSF barrier located at the choroid plexus (fig. 6.1). Amino acid transport across these barriers is carrier mediated.

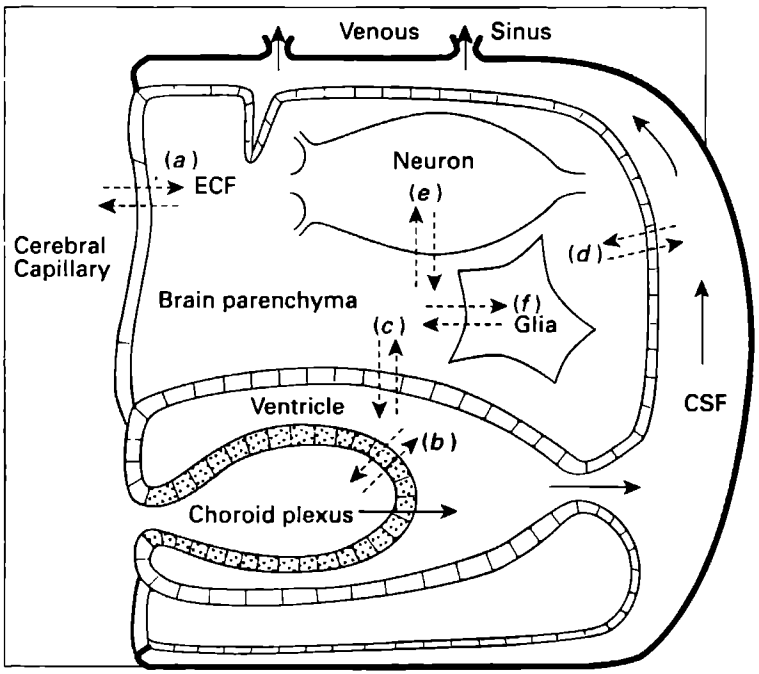

Figure 6.1 *Diagram of fluid compartments of the blood-brain-CSF system. Continuous arrows represent proven directions of CSF flow. Interrupted arrows indicate where diffusion of water and solutes may occur between the different compartments: (a) across the blood-brain barrier, between brain capillaries and extracellular fluid; (b) across the epithelia of the chroroid plexuses; (c) across the ependyma; (d) across the piaglial membranes; (e) and (f) across the cell membranes of neurons and glial cells. Thick outline represents the arachnoid-dural enclosure of the system. Illustration from [34].*

Four amino acid carriers are active at the BBB [13, 14]. The L-system (transporting leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophane, methionine, histidine, threonine, and DOPA), and the y^+-system (transporting the basic amino acids arginine and lysine), are located at the luminal side of the brain capillary endothelium, they are sodium independent and are responsible for the influx of amino acids.
The A-system (transporting the small neutral amino acids alanine, glycine, proline and methyl-aminoisobutyric acid), and the X_{AG}-system (transporting the acidic amino acids glutamic acid and aspartic acid) are located at the abluminal side of the brain capillary endothelium. They are ATP-driven and responsible for brain amino acid efflux, against a concentration gradient. The uptake of glutamine, studied in rat brain microvessels, was partly sodium dependent (A-system) and partly sodium independent (L-system) [15].
Within the brain parenchyma numerous amino acid carriers (more than 14) are described to be active at the cell-membranes [16, 17]. These carriers affect the brain extracellular fluid (ECF) composition and indirect the CSF composition, because CSF is in free exchange with the ECF. The K_m and V_{max} values for BBB transport of amino acids are 1/3 and 1/10 of values at the brain cell membrane, respectively [18]. This indicates that transport restriction is located at the BBB and not at the brain cell membrane.
At the choroid plexus all types of the mentioned carriers are shown to be present. Uptake was found to serve mainly in situ protein synthesis, meaning that efflux via the choroid plexus is probably not very important for amino acids [19].
In case of aspartic acid and glycine the flux reverses from CSF to blood when concentrations are raised [20].

Our findings in plasma amino acids in infants with CRF are in agreement with reports in literature concerning adults and children [6-8, 10, 21]. Explanations for the derangements in plasma amino acids are offered by several of the authors referred to [22-29].

No CSF studies have been performed in children with chronic renal failure before, except our own study of purines and pyrimidines in CSF of uremic children [11]. Concerning amino acids, CSF studies have been performed only in adults with chronic renal failure [2, 10].

The results we obtained in the plasma of our patients are only partly responsible for the aberrations found in CSF. In case of the large neutral amino acids we found a decrease in plasma and in CSF for valine, isoleucine, leucine, tyrosine and histidine. Decreased plasma concentrations may lead to a decreased transport via the L-carrier to the cerebral compartment. Phenylalanine, methionine, and threonine which are transported by the same carrier however, show normal values in both plasma and CSF. These findings form a strong arguement against the hypothesis of an isolated carrier-transport abnormality in CRF. Moreover, in plasma we found deviations of compounds which are not changed significantly in CSF: serine is decreased, aspartic acid and citrulline are elevated. On the contrary, for α-aminobutyric acid, isoleucine and n-ϵ-methyl-l-lysine we found isolated decreases in CSF.

In our three patients receiving CAPD only 3-methylhistidine shows clearly improved values in both compartments indicating that this particular abnormality in CSF of uremic patients is a plasma related phenomenon. So far there are no indications that during CAPD the other deviations in CSF are improving.

Changes of CSF amino acid concentrations, unrelated to alterations in their blood concentrations, have been described in adults with CRF [10]. Low γ-aminobutyric acid levels, high glycine levels, and elevated ratios of phenylalanine/tyrosine and of glycine/branched-chain amino acids have been described [1,2,9]. Pye et al. [10] extensively studied amino acids of CSF of adults with renal failure. We confirm their findings concerning the decreased concentrations of valine, leucine, and tyrosine, and the elevated concentration of 3-methylhistidine in CSF. For glycine and ornithine, for which they found elevated concentrations in CSF, we found normal values in CSF of infants with renal failure. Sullivan et al. found elevated CSF tryptophane levels and normal CSF tyrosine levels [27], for both substances we found a decrease. In case of GABA we found in four patients values above P97.5, for the whole group however the concentration was not significant elevated.

In our view intracellular disturbances are contributing to the changes found in CSF. The changes in CSF amino acids can not be explained sufficiently by affected transport systems but could arise from local - cellular - disturbances. It has been pointed out that in CRF an excess of parathyroid hormone results in alterations in membrane phospholipids

and in an incraesed intracellular calcium content [30]. There is an abundance of evidence that in chronic renal failure there is a reduced Na-K pump flux [31, 32]. This reduction has also been demonstrated in brain synaptosomes resulting in a decreased extrusion of sodium and intracellular increase of calcium [30, 33].

Studies of cultured astrocytes and neurons in uremic environment will further elucidate the metabolic alterations of these cells in CRF.

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Chapter 7

CEREBROSPINAL FLUID AMINO ACIDS, PURINES AND PYRIMIDINES AS A TOOL IN THE STUDY OF METABOLIC BRAIN DISEASES

Chapter 7

Cerebrospinal fluid amino acids, purines and pyrimidines as a tool in the study of metabolic brain diseases

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7.1 Summary

After establishing more extended reference values for amino acids, purines and pyrimidines in cerebrospinal fluid (CSF) in infancy and childhood, we studied 1250 CSF-aliquots from patients who were undergoing a diagnostic lumbar puncture for diverse clinical indications. Our primary aim was to answer the question whether determination of the concentration of amino acids, purines and pyrimidines in CSF is a useful tool in screening for metabolic disorders in children with unexplained mental retardation.
In unexplained mental retardation (95 patients) we observed varying abnormalities of CSF. These were reproducible in only 2 patients (a decrease of homocarnosine in combination with two unidentified compounds). Striking abnormalities in pyrimidine content which are limited to CSF are found in argininosuccinic aciduria and uraemia. In uraemia a general decrease in amino acids in CSF and increase of γ-aminobutyric acid (GABA) was observed.
The results obtained indicate that determination of amino acids, purines and pyrimidines in CSF is only of limited value in the diagnosis of unexplained mental retardation.

7.2 Introduction

Metabolic brain diseases can be associated with abnormal cerebrospinal fluid (CSF) levels of amino acids, purines and pyrimidines. In some disorders, like Leigh's syndrome [20] and non-ketotic hyperglycinaemia [17], the presence in CSF of high concentrations of specific metabolites (lactate and glycine, respectively) is a diagnostic feature. In argininosuccinic aciduria (ASA), a urea cycle defect, the concentration of argininosuccinic acid and its anhydrides in CSF is found to be twice to four times that noted in plasma [18] (McKusick 20790). Furthermore, it is evident that neurotransmitter metabolism shows abnormalities limited to CSF, e.g. disorders in γ-aminobutyric acid (GABA) metabolism are reported with either increased or decreased CSF levels of GABA [14].
The availability of new and more sensitive methods for determining amino acids, purines and pyrimidines urged us to establish more extended normal values of these compounds in CSF [5, 6]. Our aim was to study CSF amino acid, purine and pyrimidine levels in

various neurological disorders in order to contribute to the aetiologic unravelling of metabolic brain diseases.

We studied amino acids, purines and pyrimidines in CSF of a large number of CSF-aliquots (1250) from patients who were undergoing a diagnostic lumbar puncture for diverse clinical indications.

7.3 Materials and methods

Samples and patients

Specimens of CSF (1250) were obtained from 1154 different subjects ranging in age from 0-18 years, who were undergoing a diagnostic lumbar puncture for conventional clinical indications such as suspected central nervous system infection or other neurological disorders. Whenever possible, 1 ml of each CSF sample was put aside for the present investigation. The CSF samples were kept frozen at -70° C until analysis. Just before analysis the samples were thawed and deproteinized according to previous published methods [6].

Table 7.1 *The diagnostic groups in the patients (n = number of patients).*

		n
1.	Reference group normal children	122
2.	Rest group	265
3.	Diagnostic groups:	
	metabolic disorders	27
	heredodegenerative disorders	31
	structural congenital disorders of the brain	27
	perinatal pathology	49
	febrile convulsions	32
	epilepsy	134
	unexplained mental retardation	180
	mental retardation with a known cause	35
	syndromes	17
	infections such as meningitis	36
	oncology such as leukaemia	134

The subjects were divided into different clinical diagnostic groups by retrospective study of the patient records (table 7.1). The total number of patients studied is 856, because we had to exclude retrospectively a number of patients owing to too high erythrocyte counts in CSF or technical problems concerning the amount of CSF. Some patients had more than one diagnosis. Ninety-five patients were selected with mental retardation without any other diagnosis. The values obtained were compared with previously established normal values of amino acids, purines and pyrimidines [5, 6]. Our reference group has been extended compared to our earlier publications, the reference ranges have been confirmed and remain the same.

In addition the coefficient of variability was calculated from repeated sampling of CSF from a group of 13 patients with stable leukaemia without central nervous system localization (table 7.2).

Table 7.2 The coefficient of variability for the different compounds in CSF (n = 13).

taurine	10.5	isoleucine	11.4
phosphoethanolamine	11.6	leucine	9.8
aspartic acid	22.2	tyrosine	15.8
threonine	18.8	phenylalanine	6.9
serine	12.3	γ-aminobutyric acid	28.0
asparagine	11.7	ethanolamine	12.1
α-aminoadipic acid	30.2	tryptophane	27.2
glycine	17.5	ornithine	19.1
alanine	13.5	lysine	12.8
citrulline	13.2	histidine	14.4
α-aminobutyric acid	20.2	n-ε-methyl-l-lysine	18.4
valine	10.1	3-methylhistidine	22.1
methionine	20.2	homocarnosine	14.9
cystathionine	23.7	arginine	15.4

Analyses

For detailed information about the chemical analyses the reader is referred to previous publications [5, 6].

Statistics

The probability that a given number of subjects falls outside the normal values (defined as the interval between the 2.5 and 97.5 percentile) is calculated using a binomial distribution. The significance level is set as 0.01.

7.4 Results

Three groups showed abnormalities in either amino acids and/or purines and pyrimidines.

From the group of 'metabolic diseases' we found abnormalities in 3 patients with ASA. Besides the known deviations in amino acids (increases in argininosuccinic acid, its anhydrides and citrulline) we found an elevation in CSF of pseudouridine and uridine, which was not found in urine and plasma [8].

The second group contains 10 patients, aged 2-59 months, with a chronic renal failure and a disturbed mental development. All patients showed increased concentration of pseudouridine in CSF up to 1000 times the normal value. Plasma showed concentration up to 10 times the normal value. Eight out of 10 showed a cytidine increase limited to CSF. Plasma values were normal or slightly increased. Cytidine in CSF was even greater than the respective plasma values in 8 out of 10 patients. Also a large peak of an unknown compound was detected in the pyrimidine-spectrum of each patient [7].

In the patients with chronic renal failure a significant decrease in CSF was observed for taurine, phosphoethanolamine, aspartic acid, serine, alanine, α-aminobutyric acid, valine, isoleucine, leucine, tyrosine, tryptophane, lysine, histidine, n-ϵ-methyl-l-lysine and homocarnosine ($p \leq 0.001$). A significant increase of GABA and 3-methylhistidine was observed (both $p < 0.001$).

Plasma citrulline, α-aminobutyric acid, tryptophane and cystine were significantly decreased (p-values < 0.001) while glycine concentration was increased ($p = 0.004$).

In 3 of these patients the amino acid concentrations in CSF were determined again after more than six weeks of continuous peritoneal ambulatory dialysis (CAPD) treatment. Similar results were obtained as before CAPD treatment, only 3-methylhistidine normalized.

The largest group in our study, concerns the group with mental retardation of unknown aetiology. In this group of 95 patients careful clinical selection was done to obtain a group with no other diagnosis. Within this group, 52 patients showed deviations of more than 2SD in one or more CSF amino acids, and from these 19 patients showed deviations of more than 3SD. Fifteen of this last group of 19 were asked for permission for a repeated lumbar puncture in order to investigate whether the observed abnormalities were reproducible. Nine Patients agreed. In the CSF of these patients we could not confirm the abnormalities, except for 2 patients. They showed a decrease of homocarnosine and the presence of two unknown peaks in CSF. GABA was normal. In both patients, the two hitherto unidentified peaks in the CSF-amino acid pattern were not found in serum.
Thirty-two of the 95 retarded patients showed deviations of more than 2SD in one or more CSF purine and pyrimidine concentration. From these we selected two groups: one group of 11 patients with 5SD deviations in one or more purine and pyrimidine but no typical pattern, and a second group of 22 patients with, amongst other purine and pyrimidine deviations, a cytidine elevation of more than 2SD. One patient appeared in both groups. The last group was selected because we noted that cytidine concentration was abnormal in more cases than for other purine and pyrimidine compounds. From the first group 8 patients responded on our request for a further lumbar puncture. None of them showed reproducible patterns in CSF purines and pyrimidines. Of the group with the elevated cytidine concentration 12 responded; in no patient was the cytidine elevation reproducible.

7.5 Discussion

Reference values for amino acids in CSF of young infants and children have been infrequently reported [1, 12, 11]. As far as reported, these data have been collected from measurements using ion-exchange chromatography with the ninhydrine detection system. A few investigators have used HPLC procedures with fluorimetric detection to characterize CSF amino acid profiles [4, 9]. This method, however, has a lower resolution than our technique. As a consequence of the fluorimetric detection and the

technical modifications of our method we can measure 36 compounds in one run with a tenfold increase of sensitivity compared to the conventional ninhydrine method [6].

Concerning the reference values of purines and pyrimidines in CSF it can be stated that few data are available from literature. Abnormal concentrations of some purines and pyrimidines have been reported in a few cases of disturbances in purine and pyrimidine metabolism [15, 13, 2]. Also some reports have been published on the oxypurines in CSF in relation to hypoxia and cerebral ischaemia [16, 10].

In our patients with ASA we demonstrated aberrations in pyrimidines limited to CSF [8]. We were particularly interested in the CSF concentrations of pyrimidines, because the urea cycle is present in brain [19]. Therefore, CSF could be a sensitive indicator fluid in which to detect urea cycle defects distal to carbamylphosphate synthetase (E.C. 2.7.2.2.) by elevated pyrimidine concentrations. In ASA we demonstrated elevated concentrations of uridine and pseudouridine limited to CSF. Pseudouridine, a t-RNA catabolyte and metabolic end product, is associated with tissue destruction.

Our results in patients with chronic renal failure are striking [7]. Patients with uraemia are prone to develop a uraemic encephalopathy. Our results show that there are metabolic aberrations in uraemia limited to the cerebral compartment. The concentrations of cytidine in CSF are in our patients even greater than the plasma concentration. This could possibly give some clues for the still not understood uraemic encephalopathy. Cytidine is involved in membrane metabolism as has been pointed out [7]. We demonstrated that CAPD had no influence on these elevated concentrations.

An impaired transport of amino acids can explain the lowered concentration of most amino acids in CSF [3].

The screening for metabolic disorders in patients with unexplained mental retardation revealed only 2 patients with reproducible abnormalities in CSF amino acids. These patients showed a decrease of homocarnosine and two unknown peaks limited to CSF. The bulk of patients with retardation showed no reproducible abnormalities in either amino acids, or purines and pyrimidines. Probably, when we are able to describe different clinical subgroups in mental retardation, it will also be possible to discern statistically significant abnormalities, e.g. as we are able to describe abnormalities in amino acids in uraemia.

In our view CSF can be used to study metabolic diseases suspected to be limited to the cerebral compartment. For screening of mental retardation an additional value is only present for a small number of patients. In view of the large number of retarded children studied the results are rather poor. This is of particular importance since in mental retardation an increasing number of clinicians is requiring metabolic screening of CSF.
In this article we have given an overview of our results in using CSF amino acid and purine and pyrimidine contents as tools in the study of metabolic brain diseases. In mental retardation, clinical and biochemical findings should point to a specific cerebral metabolic involvement before CSF investigation of amino acids, purines and pyrimidines should be performed.

7.6 References

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Chapter 8

SUMMARY, GENERAL DISCUSSION AND PERSPECTIVES

Chapter 8

Summary, general discussion and perspectives

8.1 Summary and general discussion

The aim of this thesis is to explore the significance of analysis of cerebrospinal fluid (CSF) on amino acids, purines and pyrimidines in the diagnostic approach of patients with unexplained mental retardation and/or brain disease.

CSF is the clinician's access to the brain. Anatomical relations in the brain compartment, transport processes across the different barriers and other determinants influencing CSF production and composition are described in **chapter 1**. CSF is used as a diagnostic medium in central nervous system diseases such as meningitis, malignancies and metabolic disorders. In some metabolic disorders, like Leigh's syndrome [1] and non-ketotic hyperglycinemia [2], the presence in CSF of high concentrations of specific metabolites (lactate and glycine, respectively) is a diagnostic indicator. Neurotransmitter metabolism shows abnormalities limited to CSF, e.g. disorders in gamma-aminobutyric acid (GABA) metabolism are reported with either increased or decreased CSF levels of GABA [3].

In our laboratory new sensitive methods came available for determining purines and pyrimidines, and amino acids in body fluids [4,5]. These developments encouraged clinicians to determine purines, pyrimidines, and amino acids in CSF of patients for an increasing scala of indications. Especially in case of unexplained mental retardation an increasing number of CSF specimens has been investigated on these metabolites. Interpretation of investigations required knowledge of age-related reference values. These facts urged us to establish normal values of purines, pyrimidines, and amino acids in CSF of children of different ages. The methods and results of these studies are described in **chapter 2** for purines and pyrimidines [4], and in **chapter 3** for amino acids [5].

We studied 1250 CSF-aliquots from patients, who had received a diagnostic lumbar puncture for diverse clinical indications.
The subjects were divided into different clinical diagnostic groups by retrospective study of the patient records. The total number of patients studied is 856, because we had to exclude retrospectively a number of patients due to increased erythrocyte counts in CSF

or technical problems concerning the amount of CSF. The groups which showed abnormalities in either amino acids and/or purines and pyrimidines are described in the following chapters.

In **chapter 4** three patients are presented with an argininosuccinic aciduria (ASA) [6]. All three patients showed elevated concentrations of argininosuccinate and its anhydrides in all body fluids, most pronounced in cerebrospinal fluid. Moreover, in patients 1 and 2, we found elevated concentrations of pseudouridine and uridine limited to CSF, which was not reported before. In patient 3, with some residual activity of argininosuccinate lyase (ASL), we found normal values of the latter compounds. In urine we found elevated concentrations of uracil in patients 1 and 2, and orotic acid in patient 2. Plasma showed an elevated concentration of orotic acid in all three patients, uracil was elevated in patient 2, cytidine was elevated in patient 2 and 3. The results indicate that CSF values of pyrimidines reveal, so far unknown, biochemical abnormalities of brain tissue in a urea cycle disorder. The brain tissue has its own urea cycle.

In children with chronic renal failure (CRF) abnormalities in CSF pyrimidines have been detected, too [7]. These findings are presented in **chapter 5**, and point to a metabolic disturbance in CRF limited to the cerebral compartment.
Chronic renal failure during childhood can be associated with delayed cognitive development. From ten children with chronic renal failure, aged 2-59 months, plasma and (CSF) purines and pyrimidines have been determined. A marked increase of pseudouridine and cytidine was demonstrated in CSF of ten and eight children, respectively. The plasma concentration of pseudouridine was increased in a varying degree to a maximal value of more than ten times the upper limit of normal. The plasma concentration of cytidine showed only moderately elevated values. In three children the study in CSF and plasma was repeated 6 weeks after the start of continuous ambulatory peritoneal dialysis. The abnormal concentrations of pseudouridine and cytidine were still present in CSF and plasma. From these findings it was concluded that this disturbance has implications for the cell metabolism as explained in chapter 5.

Further studies are necessary to elucidate the cause of this unknown biochemical aberration of the central nervous system.

In chapter 6 we describe our findings of amino acids in children with CRF [8]. Plasma and CSF amino acids concentrations were determined in 8 infants (age 2-8 months) and 3 children (age 26, 32 and 56 months) with CRF (creatinine clearance 13 ± 9 ml/min/1.73m2). In three of these children investigations were repeated after six weeks of CAPD treatment.
In the infants, a significant decrease was found in CSF of α-aminobutyric acid, valine, isoleucine, leucine, tyrosine, tryptophane, histidine and n-ε-methyl-l-lysine, whereas there was a significant increase of 3-methylhistidine. In plasma serine, valine, leucine, tyrosine, and histidine were significantly decreased, whereas there was a significant increase of aspartic acid, citrulline, and 3-methylhistidine.
These abnormalities remained constant after the start of CAPD except for the normalisation in CSF and plasma of 3-methylhistidine.
These data indicate a generalized disturbance of amino acids in young children with CRF. An abnormal substrate is offered to the neurons and astroglia in children with CRF. The results can not be explained by disturbed transport only, and demonstrate that there is a disturbance at the cellular level.

Chapter 7 describes our efforts to answer the question whether determination of the concentration of amino acids, purines and pyrimidines in CSF is a useful tool in screening for metabolic disorders in children with unexplained mental retardation [9].
In unexplained mental retardation (95 patients) we observed varying abnormalities of these metabolites in CSF. These deviations, however, were reproducible in only 2 patients (a decrease of homocarnosine in combination with two unidentified compounds). The obtained results indicate that determination of amino acids, purines and pyrimidines in CSF is of limited value in the diagnosis of unexplained mental retardation.

When comparing chapters 6 and 7 one may notice differences in the results of the amino acids in CSF in the same group of children with CRF. This is due to the fact that the

results in chapter 7 were preliminary at the time of publication. Statistics were performed using a binomial distribution. Later on we were able to use Student's t-test, which resulted in better but slightly different results.

8.2 Further research; brain-specific proteins

With reference to chapters 5 and 6, it can be stated that further investigation of the genesis of uremic encephalopathy is of utmost importance for the developing child with CRF. Our work provides starting points for this research. The nature of the 'unknown' peaks should be revealed. Specialists in mass-spectrometry, here is your task.

We performed a study in which the brain-specific proteins in CSF of children with CRF have been determined. We studied the CSF-levels of neuron-specific enolase (NSE), S-100 and myelin basic protein (MBP) in order to identify the affected cell type or compartment. The same patients as described in chapter 6 [8] were studied. Increased CSF levels of NSE are indicative for neuron damage, S-100 for astroglial cell damage and MBP for demyelinisation [10].
The brain-specific proteins were determined according to previously published methods including the reference values for children as established in our laboratory [11].
Brain-specific proteins were determined in all patients. We found elevated levels of S-100 in 5 patients: patient no. 3, 2.8 μg/l; no. 5, 2.7 μg/l; no. 8, 6.8 μg/l; no. 10, 2.8 μg/l; and no. 11, 3.3 μg/l (normal 0.9-2.6 μg/l). The remaining 6 patients showed values within the normal range. MBP and NSE showed normal values, except for patient 8, who showed a MBP of 1.4 μg/l (normal 0.12-0.72 μg/l). The three (nrs. 2, 6, and 9, see chapter 6) patients who underwent a second lumbar puncture after more than six weeks of CAPD, had by coincidence normal levels of the brain-specific proteins from the start, so we are not able report whether there are possible effects of CAPD on elevated brain-specific proteins.

The observation of abnormal concentrations of the brain-specific protein S-100 in 5 out of 11 patients points to the astroglial cell as an affected cell type in cerebrum in CRF. In

patient 8, showing the highest S-100 level, a concomittant elevated MBP content was demonstrated, suggesting also demyelinisation.
Our findings are in agreement with suggestions made in literature: La Greca et al. [12] suggest that glial alterations could result in BBB alterations and subsequent metabolic alterations. The astroglial cell is responsible for BBB integrity, and for the nourishing of neurons. It is the main site where glutamate is converted into glutamine [13,14]. An affected astroglial cell compartment could result in altered BBB characteristics, and altered transport within the astroglial cell itself affecting the nourishing of neurons.

8.3 Perspectives; nuclear magnetic spectroscopy

With respect to the difficulties experienced for repeating the lumbar puncture non-invasive techniques become important. From a research point of view it is important to know what is happening in vivo inside the uremic brain. Moreover, in the children with CRF we are interested in monitoring therapy in a noninvasive way, especially when a better understanding of uremic encephalopathy can lead to improvement of therapy.
Nuclear magnetic resonance (NMR) spectroscopy provides the possibilities to answer at least partially these needs [15]. NMR spectra of human brain in vivo reveal a large number of components which are of major relevance for the metabolism of the functioning brain [16]. Enhanced levels of cerebral glutamine are detected in patients with liver cirrhosis [16,17]. Reductions of choline metabolites and myo-inositol levels have been reported in hepatic encephalopathy as well [17]. Studies of four patients with uraemia are reported in litterature. They showed a tendency towards higher choline and myo-inositol concentrations, but the results did not achieve statistical significance [17]. Suggestions have been made in literature that hepatic encephalopathy and uraemic encephalopathy are based on similar pathophysiological mechanisms [18].
We performed NMR spectroscopy in two children with CRF. These spectra showed a higher myoinositol level in 1 patient and small differences in glutamine and glutamic acid peaks in both patients compared to a control (fig 8.1.). This is in line with the discussion above.

Further research in this direction has to be performed, especially in young children with CRF, which are more vulnerable to metabolic changes during the maturation of the brain.

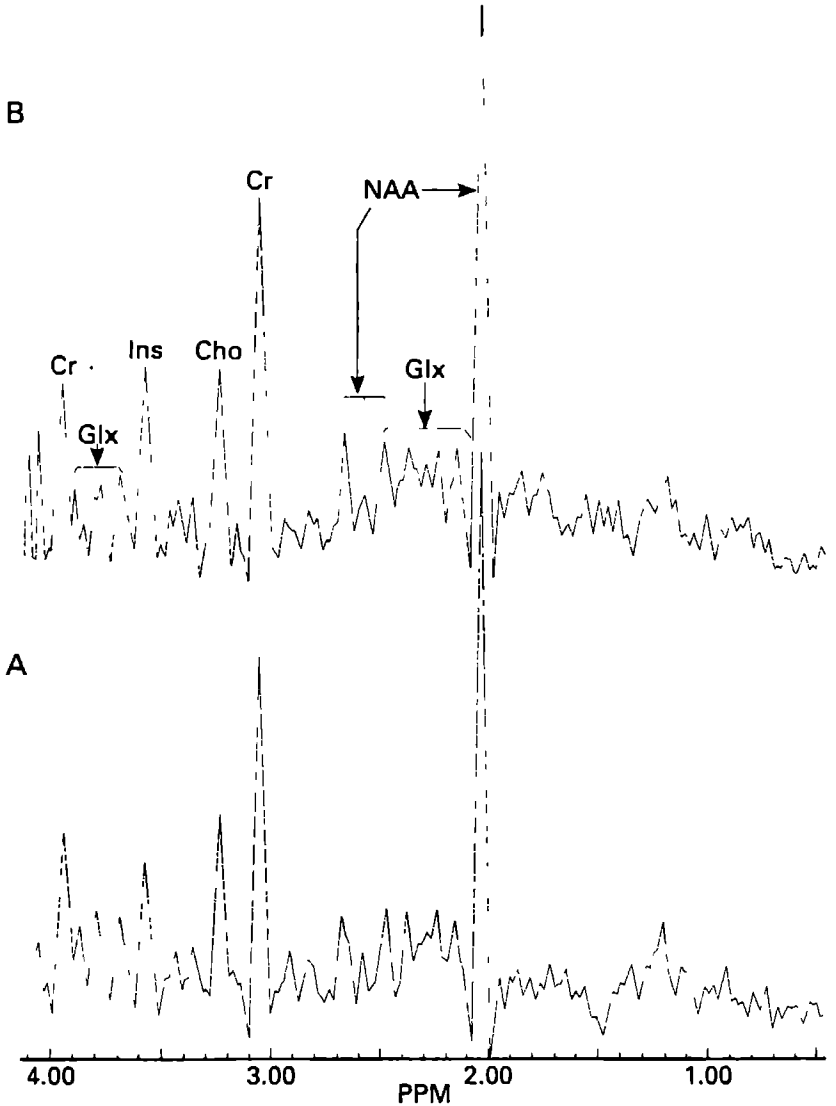

Figure 8.1 *Cerebral H-1 MR spectra demonstrating the major differences between a patient (B) with CRF and a control (A). Te abbreviation NAA denotes n-acetylaspartate; Ins, inositol; Cho, choline; Cr, creatine; and Glx, glutamine and glutamate complex.*

8.4 Microdialysis of brain extracellular fluid

In our study of the value of CSF in metabolic brain derangements, we interprete CSF as a reflection of brain extracellular fluid (ECF). In chapter 6 we hypothesize that intracellular disturbances are contributing to the changes found in CSF of uremic children. To achieve a better understanding of the factors involved it would be a progress to measure exactly the ECF concentrations of different compounds involved. To measure directly in ECF microdialysis techniques are required and available now [19]. This technique will allow us to study the composition of the extracellular fluid at different localisations in the brain. Investigation of the uremic state in this way may offer new perspectives.

8.5 References

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Samenvatting

Het doel van dit proefschrift is te onderzoeken wat de waarde is van de bepaling van aminozuren, purines en pyrimidines in de liquor cerebrospinalis in het kader van de diagnostiek bij patiënten met een onbegrepen mentale retardatie of cerebrale aandoening.

De liquor is voor de klinicus de toegangsweg tot de hersenen. Anatomische relaties binnen het hersenkompartiment, transport-processen over de verschillende barrières en andere faktoren welke een rol spelen bij de liquorproduktie en samenstelling daarvan worden beschreven in **hoofdstuk 1**. Liquor wordt gebruikt als een diagnostisch medium bij aandoeningen van het centraal zenuwstelsel zoals meningitis, maligniteiten en metabole ziekten. Bij sommige metabole aandoeningen, zoals bij het syndroom van Leigh [1] en bij de non-ketotische hyperglycinemie [2], is de bevinding van een verhoging van specifieke metabolieten (respektievelijk laktaat en glycine) een belangrijk diagnostisch kriterium. Het neurotransmitter metabolisme laat verstoringen zien welke alleen in liquor aantoonbaar zijn; ontregelingen in het metabolisme van gamma-aminoboterzuur bijvoorbeeld worden beschreven met zowel verlagingen als verhogingen van de liquorkoncentratie van het vrije gamma-aminoboterzuur [3].

In ons laboratorium zijn nieuwe sensitievere methoden tot onze beschikking gekomen voor het bepalen van aminozuren, purines en pyrimidines in lichaamsvloeistoffen [4,5]. Deze ontwikkelingen nodigen klinici uit tot het aanvragen van bepalingen van deze stoffen in liquor in een groeiend indicatiegebied. Met name in geval van de onbegrepen mentale retardaties wordt er een toenemend aantal liquormonsters op deze metabolieten onderzocht. Interpretatie van deze bepalingen vereist de beschikking over goede leeftijdsgebonden referentiewaarden. Deze faktoren noopten tot het vaststellen van referentiewaarden voor aminozuren, purines en pyrimidines in liquor voor verschillende leeftijdsgroepen. De methoden en resultaten worden voor wat betreft referentiewaarden van de purines en pyrimidines beschreven in **hoofdstuk 2** [4], en voor de aminozuren in **hoofdstuk 3** [5].

Onderzocht werden 1250 liquormonsters van patiënten, die op klinische gronden een lumbaal punktie ondergingen.
De patiënten werden retrospectief onderverdeeld in verschillende diagnostische groepen. Het totaal aantal bestudeerde patiënten bedraagt 856, daar er retrospectief een aantal moesten worden uitgesloten ten gevolge van bijvoorbeeld een te hoog erythrocytengehalte in de liquor of technische problemen bij de bepaling. Daarnaast onderging een aantal patiënten seriële punkties. De patiëntengroepen welke afwijkingen vertoonden in aminozuren en/of purines en pyrimidines worden beschreven in de volgende hoofdstukken.

In **hoofdstuk 4** worden drie patiënten beschreven met een argininosuccinaat-acidurie [6]. Alle drie hebben ze een verhoging van het argininosuccinaat en de anhydriden in urine, plasma en de liquor. Daarnaast vonden we bij patiënten 1 en 2 alleen in de liquor verhoogde koncentraties van pseudouridine en uridine. Dit is niet eerder beschreven. Bij patiënt 3, welke nog wat restaktiviteit bezit van het argininosuccinaat-lyase, vonden we normale koncentraties van deze stoffen. In de urine werden bij patiënten 1 en 2 verhoogde koncentraties aangetroffen van uracil, en bij patiënt 2 eveneens een verhoogde koncentratie van orootzuur. Het plasma liet verhoogde koncentraties zien van orootzuur bij alle drie patiënten, uracil was verhoogd bij patiënt 2, cytidine was verhoogd bij patiënten 2 en 3. De resultaten geven aan dat de liquorwaarden van pyrimidines nieuwe, tot nu toe onbekende, biochemische afwijkingen laten zien in hersenweefsel bij een stoornis in de ureumcyclus. De hersenen hebben een eigen ureumcyclus.

Bij kinderen met een chronische nierinsufficiëntie werden eveneens afwijkingen vastgesteld bij de pyrimidines in de liquor [7]. Deze bevindingen, die gepresenteerd worden in **hoofdstuk 5**, wijzen op een metabole verstoring bij de chronische nierinsufficiëntie welke beperkt is tot het cerebrale kompartiment.
Chronische nierinsufficiëntie bij kinderen wordt geassocieerd met een vertraagde cognitieve ontwikkeling. Bij tien kinderen met een chronische nierinsufficiëntie, in leeftijd variërend van 2 tot 59 maanden, werden purines en pyrimidines bepaald in plasma en liquor. Een opvallende verhoging van het pseudouridine werd aangetoond bij alle tien kinderen, en van cytidine bij acht kinderen. De plasmakoncentratie van pseudouridine was

wisselend verhoogd, soms tot tienmaal de bovengrens van normaal. De plasmakoncentratie van cytidine liet slechts minimale verhogingen zien. Bij drie kinderen werd het onderzoek van liquor en plasma herhaald zes weken na de start van peritoneaaldialyse. De abnormale koncentraties van pseudouridine en cytidine waren onveranderd in zowel liquor als plasma. Deze bevindingen hebben implikaties voor het celmetabolisme zoals beschreven wordt in hoofdstuk 5.
Vervolgstudies zijn noodzakelijk om de oorzaak aan te tonen van deze onbekende metabole verstoring van het centraal zenuwstelsel.

In **hoofdstuk 6** beschrijven we onze bevindingen betreffende de aminozuren bij -inmiddels 11- kinderen met een chronische nierinsuffiëntie [8]. Aminozuurkoncentraties in plasma en liquor werden bepaald bij acht zuigelingen (leeftijd 2-8 maanden) en drie kinderen (leeftijd 26, 32 en 56 maanden) met een chronische nierinsufficiëntie (kreatinineklaring 13 $\pm$ 9 ml/min/1.73m^2). Bij drie van deze kinderen werden de onderzoeken herhaald na zes weken peritoneaaldialyse.
Bij de zuigelingen werd een signifikante daling in liquor gevonden van α-aminoboterzuur, valine, isoleucine, leucine, tyrosine, tryptophaan, histidine en n-ϵ-methyl-l-lysine, daarnaast werd er een signifikante toename in liquor gevonden van 3-methylhistidine. In plasma waren serine, valine, leucine, tyrosine, en histidine signifikant verlaagd, terwijl asparaginezuur, citrulline, en 3-methylhistidine een signifikante verhoging lieten zien.
Deze afwijkingen bleven aanwezig na het starten van peritoneaaldialyse, behalve voor 3-methylhistidine, dat normaliseerde in zowel plasma als liquor.
Deze bevindingen wijzen op een gegeneraliseerde verstoring van de aminozuren bij jonge kinderen met een chronische nierinsufficiëntie. Een afwijkend substraat wordt aangeboden aan de neuronen en astroglia bij deze kinderen. De resultaten kunnen niet verklaard worden door verstoorde transportprocessen alleen, doch geven aan dat er een stoornis is op cellulair niveau.

Hoofdstuk 7 beschrijft onze pogingen om een antwoord te geven op de vraag of de bepaling van aminozuren, purines en pyrimidines in liquor een bruikbare methodiek is bij de screening op metabole aandoeningen bij patiënten met een onbegrepen mentale retardatie [9].

Bij 95 patiënten met een onbegrepen mentale retardatie vonden we in de liquor een wisselend scala aan afwijkingen van deze metabolieten. Deze afwijkingen waren slechts bij twee patiënten reproduceerbaar (beiden hadden in liquor een verlaagd homocarnosine met daarnaast twee onbekende metabolieten). De verkregen resultaten geven aan dat de bepaling in liquor van aminozuren, purines en pyrimidines slechts een beperkte waarde heeft bij de diagnostiek van onbegrepen mentale retardaties.

Hoofdstuk 8 is een samenvattende diskussie waarbij enkele lijnen voor de toekomst worden uitgezet.

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Curriculum Vitae

Peter Gerrits werd op 8 januari 1957 te Doetinchem geboren en groeide op in Hengelo (GLD). Na het behalen van het diploma Atheneum B in 1975 aan het St. Ludgercollege te Doetinchem werd een aanvang gemaakt met de studie Geneeskunde aan de Katholieke Universiteit te Nijmegen. Tijdens de studie was hij werkzaam in verschillende bestuurlijke organen. In november 1982 werd het artsexamen afgelegd.

In 1983 was hij werkzaam op de bloedbank in Arnhem. Van januari tot oktober 1984 was hij werkzaam als arts-assistent op de afdeling Intensieve Zorg van het Academisch Ziekenhuis Nijmegen St Radboud.

Oktober 1984 werd een aanvang gemaakt met de opleiding tot kinderarts in het Canisius-Wilhelmina Ziekenhuis te Nijmegen (Opleider: Dr. P.M.V. van Wieringen). Oktober 1986 werd de opleiding voortgezet op de afdeling kindergeneeskunde van het Academisch Ziekenhuis Nijmegen St Radboud (Hoofd: Prof. Dr. G.B.A. Stoelinga). Oktober 1988 werd hij in het specialistenregister ingeschreven als kinderarts. Daarna was hij nog enige maanden als kinderarts werkzaam op de subafdeling Neonatologie.

Van juli 1989 tot april 1993 was hij werkzaam als kinderarts in het Sint Anna Ziekenhuis te Oss, en na een maatschapsfusie ook in het Sint Joseph Ziekenhuis te Veghel. Vanaf april 1993 is hij als kinderarts verbonden aan het Canisius-Wilhelmina Ziekenhuis te Nijmegen.

STELLINGEN

behorend bij het proefschrift

METABOLIC INVESTIGATION OF CEREBROSPINAL FLUID IN CHILDHOOD

amino acids, purines and pyrimidines
in brain disease

Peter Gerrits

1.

De metabole stoornissen in cerebro bij de chronische nierinsufficiëntie worden niet veroorzaakt door transportproblemen over de bloed-hersenbarrière, maar door intracellulaire processen (dit proefschrift).

2.

De bepaling van purines en pyrimidines in liquor is een veelbelovende aanvulling in het onderzoek naar de uremische encefalopathie en in de behandeling daarvan (dit proefschrift).

3.

De bepaling van aminozuren, purines en pyrimidines in liquor is als screening bij onbegrepen retardaties niet geïndiceerd en leidt tot een onverantwoorde kostenverhoging in de gezondheidszorg (dit proefschrift).

4.

De prognose van de nierfunktie bij kinderen met urethrakleppen is grotendeels reeds in utero bepaald (Parkhouse et al. Long term outcome of boys with posterior urethral valves. Br J Urol 1988;62:59-62).

5.

Het nut van NO toediening bij de behandeling van pulmonale hypertensie bij pasgeborenen dient met voortvarendheid ter hand te worden genomen (Kinsella et al. Clinical responses to prolonged treatment of persistent pulmonary hypertension of the newborn with low doses of inhaled nitric oxide. J Pediatr 1993;123:103-8).

6.

De autosomaal recessieve vorm van nefrogene diabetes insipidus kan worden veroorzaakt door een mutatie in het waterkanaal aquaporin 2, aanwezig in de verzamelbuis.

7.

Gezien de resultaten van gentherapie bij de muis met cystic fibrosis zijn de mogelijkheden van positieve resultaten bij de mens niet denkbeeldig (Tizzano and Buchwald. Recent advances in cystic fybrosis research. J Pediatr 1993;122:985-8).

8.

De weerstand tegen een lumbaal punktie wordt vooral bepaald door vóóroordelen.

9.

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10.

Neonatologen dienen rekening te houden met de mogelijkheid van een levensbedreigende erfelijke stofwisselingsziekte bij ernstig zieke pasgeborenen.

11.

Het verspreiden van het konsensusbeleid bij de asthmabehandeling bij kinderen, heeft de kwaliteit van deze behandeling aanmerkelijk verbeterd.

12.

Het behoud van het karakter van de Ooij-polder is niet alleen van belang voor de kikkers.