FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

Cerebral metabolism during acute liver failure and the effect of treatment with fractionated plasma separation and adsorption

PhD thesis

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The thesis is based on the following papers:

I: Bjerring PN, Hauerberg J, Frederiksen HJ, Jorgensen L, Hansen BA, Tofteng F, Larsen FS. Cerebral glutamine concentration and lactate-pyruvate ratio in patients with acute liver failure. Neurocrit Care. 2008;9(1):3-7.

II: Bjerring PN, Hauerberg J, Jorgensen L, Frederiksen HJ, Tofteng F, Hansen BA, Larsen FS. Brain hypoxanthine concentration correlates to lactate/pyruvate ratio but not intracranial pressure in patients with acute liver failure. J Hepatol.2010 Dec;53(6):1054-8.

III: Bjerring PN, Hauerberg J, Frederiksen HJ, Bay H, Clemmesen JO, Larsen FS. The effect of fractionated plasma separation and adsorption on cerebral amino acid metabolism and oxidative metabolism during acute liver failure. In preparation.

Abbreviations:
ALF: acute liver failure; ATP: adenosine triphosphate; BBB: blood-brain barrier; CBF: cerebral blood flow; CPP: cerebral perfusion pressure; ICP: intracranial pressure; FPSA: fractionated plasma separation and adsorption; LP: lactate to pyruvate; MAP: mean arterial pressure; MPT: mitochondrial permeability transition; TCA tricarboxylic acid;

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Preface

The work presented in this thesis was done during my appointment as a research fellow in the Department of Hepatology at Rigshospitalet and while I was enrolled as a PhD student at the University of Copenhagen in the period from 2008 to 2011. The project was conducted under the supervision of Fin Stolze Larsen. The clinical studies were done in the intensive care unit at the Department of Hepatology section 3-16-3, and the analyses made in the Laboratory of Hepatology section 2-15-1.

I would like to thank Fin Stolze Larsen, Otto Clemmesen and the Bent Adel Hansen, who was head of the department during most of my PhD, for their excellent scientific support during all phases of my work. I am also thankful for the large amount of work in the lab performed by Nine Scherling by measuring amino acid content in the never-ending row of samples I provided for her. I am grateful to the amount of work my co-authors have put into the three publications my thesis is based on.

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Aims of the studies

The primary aim of the studies was to investigate the relationship between cerebral metabolism of both carbohydrates and amino acids and the intracranial pressure of patients with acute liver failure (ALF). Moreover, the effect of treatment with the extracorporeal liver-support system, based on the principle of fractionated plasma separation and adsorption (FPSA) on cerebral metabolism was investigated.

The following studies were performed

Study I: The correlation between cerebral glutamine and lactate to pyruvate (LP) ratio was studied in patients with ALF (n=13).

Study II: The correlation between cerebral LP ratio and adenosine degradation products was studied in patients with ALF. Furthermore the relationship between brain metabolites and clinical scoring systems was evaluated (n=17).

Study III: The effect of FPSA on cerebral metabolism of amino acids and carbohydrates was studied in patients with ALF (n=7).

Background

ALF is a life threatening condition. The incidence in Denmark is around 1 per 100.000 persons per year and the survival rate is about 40-50% depending on the etiology. ALF is defined as the onset of hepatic encephalopathy (HE) within 72 days after onset of jaundice in patients with no previous symptomatic liver disease. A subgroup of ALF is termed hyperacute liver failure if the time span between first sign of liver failure and HE is less than one week. On the other hand subacute liver failure is defined as development of HE within four weeks to 72 days after onset of first symptom. The treatment of patients with ALF is most of all supportive and long-term survival is dependent on either regeneration of the failing liver or liver transplantation.

Patients with ALF carry a high risk of multi-organ failure and death. Characteristically, patients often develop severe coagulopathy, arterial hypotension due to extensive systemic arteriolar vasodilatation (1), sepsis, and renal failure. In addition, a substantial proportion of the patients develop brain edema. This is most pronounced in patients with hyperacute liver failure where brain edema is present in around 50% of the population. Brain edema is frequently accompanied with an increase in cerebral blood flow (CBF) and high intracranial pressure (ICP) (2, 3) which both carries a high risk of brain herniation and death. Although the molecular and pathophysiological mechanisms of the cerebral complications remain incompletely understood, high circulating concentrations of ammonia seems to play a pivotal role (4, 5).

Ammonia

Ammonia, NH_3, is a water-soluble compound and under physiological conditions (pH 7.40 and temperature 37°C) more than 97% of the ammonia molecules are protonated as the ammonium ion NH_4^+. The source of circulating ammonia in the human blood is primarily portally derived from glutamine metabolism in the intestinal epithelium, urease activity in the intestinal flora and renal production (6-8). Circulating ammonia can be used for glutamine synthesis in skeletal muscles and the brain, and more importantly used for urea synthesis in the liver. When the liver is failing the arterial ammonia level usually increases above the upper limit of normal of 32 µmol/L. A high arterial ammonia level leads to accumulation in the brain (9) and here ammonia exerts numerous deleterious effects contributing to the development of HE.

Blood-brain barrier and ammonia

The blood-brain barrier (BBB) is composed by the capillary endothelial cells connected by tight junctions and is closely related to the end-feet of the astrocytes outlining the pericapillary space (10). The BBB surrounds the entire central nervous system and provides a protection of the metabolic milieu in the brain from the changing concentrations of many substances in the blood. The transport of ammonia across the BBB is thought to be a combination of passive diffusion of NH_3 and transport of the ammonium ion through cation channels (11). Potassium channels are probably passable for the ammonium ion due to chemical similarities of ion size and diffusion coefficients and involved in transmembrane ammonium ion transport observed in cultured astrocytes (12). Specific ammonium ion transporters have, however, not been defined in the BBB, but theoretically, ammonium ion transporters as found in the thick ascending loop of Henle in the kidneys (13), could be present in the brain. The cerebral uptake and metabolic rate of ammonia is increased during liver failure, hyperammonemia and encephalopathy as demonstrated by positron emission tomography (14, 15) in cirrhotic patients. In ALF increased brain uptake of ammonia and efflux of glutamine has been associated with fatal intracranial hypertension (ICH) (16). A substantial amount of ammonia is accumulated in the brain by its incorporation in biochemical processes as discussed below.

Ammonia detoxification

Cerebral detoxification of ammonia takes place by incorporating ammonia in amino acid synthesis, predominantly glutamine (17). This attenuates the direct neurotoxic effect of ammonia but introduces metabolic disturbances by substrate depletion and dysequilibrium of biochemical pathways (18). Since one of the astrocytes many functions is to maintain the perineuronal environment in regard to ion and transmitter homeostasis, astrocytes play a central role in ammonia detoxification. Once ammonia has crossed the BBB, glutamine synthetase, primarily found in astrocytes (19), is amidating glutamate to glutamine by utilisation of ammonia. A shortage of glutamate is partly prevented by amination of α-ketoglutarate to glutamate (18, 20). The consequence of this is substrate depletion of the tricarboxylic acid (TCA) cycle where α-ketoglutarate is an essential intermediate. Furthermore, ammonia has been shown to inhibit two rate limiting enzymes in the pathway of glucose metabolism, namely pyruvate dehydrogenase (supplying the TCA cycle with acetyl-CoA) and α-ketoglutarate dehydrogenase (21, 22). This inhibition will slow the overall oxidative metabolism and lead to depletion of energy-rich phosphate

compounds (mainly adenosine triphosphate (ATP)) and accumulation of lactate (23), which is further discussed below. To a certain degree, the supply of intermediates in the TCA cycle can be restored by anaplerosis, i.e. energy consuming processes that bypasses the normal flow of metabolites in the TCA cycle. The primary anaplerotic pathway is thought to be carboxylation of pyruvate to oxaloacetate yielding substrates for the first steps in the TCA cycle and thereby providing carbon-skeletons for glutamate synthesis and subsequent ammonia detoxification by glutamine synthesis (20). Another anaplerotic pathway is by deamination of branched-chain amino acids (e.g. valine, leucine and isoleucine) also yielding substrates for the TCA cycle. To summarize, an important consequence of ammonia-detoxification in the brain is impaired oxidative metabolism leading to a less efficient production of energy-rich phosphate compounds measured as the number of ATP molecules produced from one molecule of glucose.

Astrocyte dysfunction

Swelling of the astrocyte is a prominent feature of encephalopathy in liver failure (24, 25). The accumulation of glutamine in the brain has been speculated to have a direct effect on the astrocyte volume. A long standing hypothesis focuses on the osmotic effects of accumulation of intracellular glutamine and the associated cellular edema (26, 27). In recent years it has also been suggested that glutamine acts as a 'Trojan horse' by operating as a carrier of ammonia and transport it from the cytosol to the mitochondria (28). In the mitochondria ammonia both induces oxidative and nitrosative stress by formation of free radicals and this in turn leads to the pre-apoptotic process called mitochondrial permeability transition (MPT) (29). MPT is characterized by a sudden loss of inner membrane potential and cessation of mitochondrial ATP synthesis. Other inducers of MPT are high intracellular Ca^{2+} levels (as seen after excitotoxicity) and alterations in mitochondrial redox-state and pH (30). The occurrence of MPT in cultured astrocytes during ammonia exposure has reported to be associated to increased cell volume (31). Although MPT has been extensively investigated due to its involvement in apoptosis and many disease states (e.g. traumatic brain injury, degenerative neurological diseases and myocardial ischemia-reperfusion) the pore-complex in the mitochondrial inner membrane responsible for the sudden increase in permeability has not been structurally identified (32).

Cerebral glucose metabolism

The human brain has an energy consumption of approximately 20 watt or around 20% of the total energy consumption of the human body in a resting state. Most of this energy is used for reversing the ion influxes of the neuronal cells that are a result of synaptic activity and action potentials. Almost all energy is derived from glycolysis, i.e. oxidation of glucose to pyruvate and furthermore oxidation of pyruvate to carbon dioxide by the TCA cycle under aerobic conditions. This implies that a constant delivery of glucose and oxygen to the brain has to be maintained. In brain tissue of healthy humans, roughly 165 μmol/100 g*min of oxygen and 30 μmol/100 g*min of glucose is metabolised. This equals to approximately 53 grams of oxygen and 110 grams of glucose per day for the brain of an adult. Under anaerobic conditions glycolysis is the only source of ATP and in order to regenerate NAD^+ used in glycolysis, pyruvate is dehydrogenated to lactate (Figure 1). Hence, if the supply of oxygen is insufficient or the mitochondria are not functioning properly, lactate levels increases and pyruvate levels decrease. Consequently, an increase in the lactate to pyruvate (LP) ratio is seen, and an elevated LP ratio is indeed recognised as a sensitive indicator of compromised oxidative metabolism.

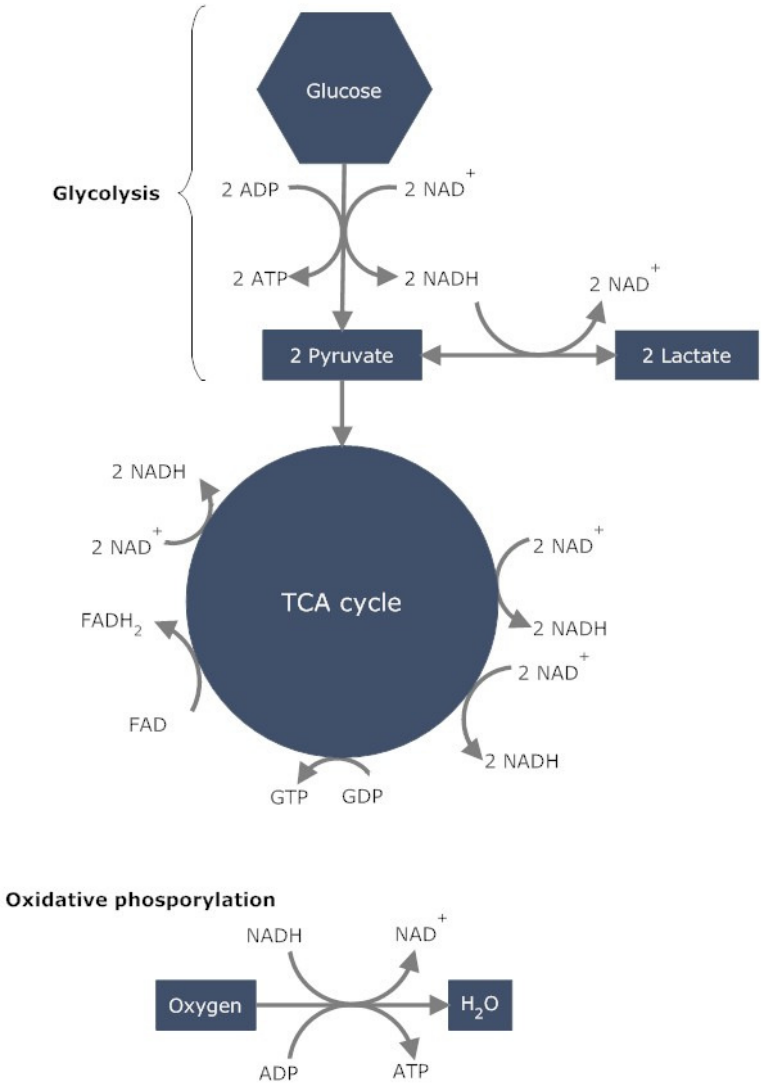

Figure 1 - The main features of glucose metabolism. Glycolysis takes place in the cytosol and the tricarboxylic acid (TCA) cycle in the mitochondrion. The oxidative phosphorylation takes place in the mitochondrial inner membrane by the enzyme complexes of the respiratory chain. During oxidative metabolism the glucose degradation leads to the production of the reducing equivalents NADH and $FADH_2$ that are reoxidized with the generation of ATP and water by oxidative phosphorylation. If not sufficient oxygen is supplied, or if the respiratory chain is not functional (i.e. as a consequence of mitochondrial permeability transition) the regeneration of NAD^+ during oxidative phosphorylation will cease. Then the TCA cycle will be inhibited by the lack of NAD^+ and the ATP generation will only rely on glycolysis. NAD^+ regeneration is in that case done by conversion of pyruvate to lactate which leads to an increase in the lactate to pyruvate ratio.

ATP degradation and hypoxanthine

During situations with an impaired ATP synthesis, intracellular levels of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) rise due to dephosphorylation in intermediary metabolism. Furthermore, studies have shown that hypotension, hypoxia and certain types of cellular stress, lead to cellular release of ATP and adenosine to the intracellular compartment (33-35). Receptors sensitive to ATP and adenosine, i.e. purinoceptors, are found in most tissues (36) and it is speculated that purinoceptors are mediating paracrine signals related to regulation of cerebral microcirculation (37, 38), apoptosis (39, 40) and endogenous neuroprotection (41). In the extracellular compartment, ATP, ADP, and adenosine are rapidly degraded by extracellular dephosphorylation and deamination that leads to formation of inosine, which in turn is deribosylated by purine nucleoside phosphorylase to hypoxanthine. Hypoxanthine is converted to uric acid by xanthine oxidase in an oxidative environment. The line of processes leading from ATP to uric acid during impaired oxidative metabolism and extracellular release can be simplistically summarised as:

ATP → ADP → AMP → adenosine → inosine → hypoxanthine → uric acid

Hypoxanthine found in both cerebrospinal fluid, microdialysate, and arterial plasma has been found a significant marker of cerebral hypoxia and hypoperfusion (42-44) as well as in the brain of patients with traumatic brain injury (45).

Cerebral amino acid transport

The brain is dependent on uptake of essential amino acids for protein synthesis. The BBB is contributing actively to maintaining an amino acid homeostasis in the brain by a complex transport system in endothelial cells (Figure 2).

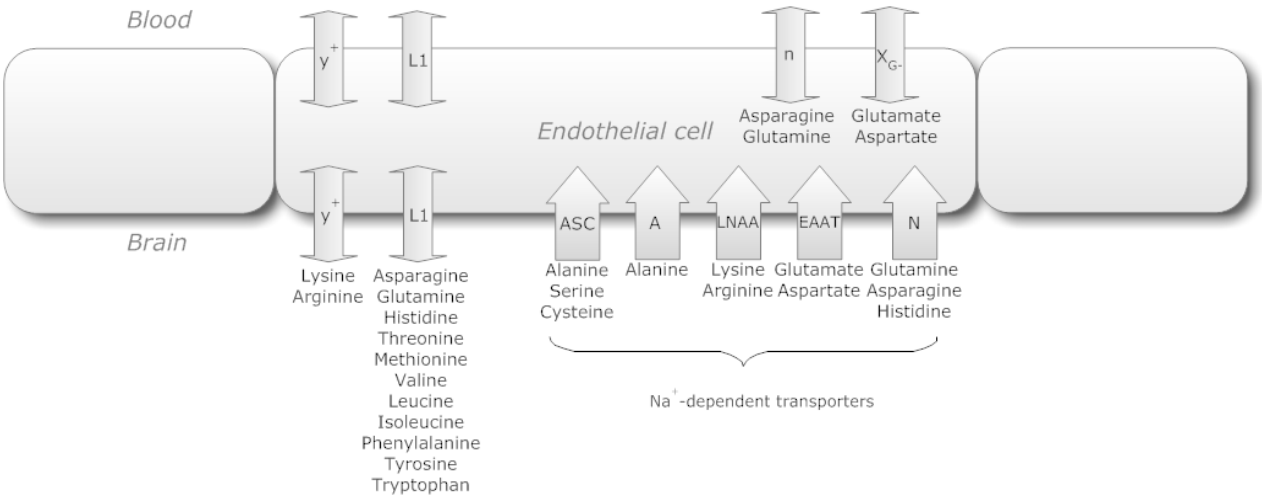

Figure 2 - Transport of amino acids across the blood-brain barrier. The preferred amino acids for each transporter is stated below the transporter symbols. The facilitative (bidirectional) transporters y^+, L1, n, and X_{G^-} under normal circumstances provide an influx of amino acids whereas the Na^+-dependent (unidirectional) transporters ASC, A, LNAA, EAAT, and N provides an efflux. The figure is based on (46).

In brief, facilitative transporters (L1 and y^+) in both the luminal and abluminal endothelial cell membranes provide an influx of essential amino acids (46). Furthermore, a Na^+-dependent transport system exists in the abluminal membrane. The Na^+-dependent system consists of at least five transporters (ASC, A, LNAA, EAAT and N) that facilitate movement of amino acids against a concentration gradient from the extracellular compartment of the brain to the cytosol of the endothelial cells and subsequently to the capillaries. This is indirectly an energy consuming process that relies on the Na+/K+-ATPase maintains a concentration gradient of Na+ - i.e. secondary active transport by an antiport. The transport system is redundant, as each transporter has affinity for one or more amino acids and each amino acid is a substrate for one or more transporters in both the luminal and abluminal membrane. In healthy subjects this ensures a concentration of most amino acids in the extracellular compartment of the brain is kept at approximately 10% of the arterial concentration, except of the glutamine concentration that normally is around 85% of the arterial concentration. The amino acid transporters in the BBB demonstrate saturation kinetics, for example

the Km is approximately 1 mmol/L of the N transporter that transports glutamine out of the brain (47), which means that the maximal rate of glutamine transport is reached when the extracellular level reaches ~2 mM – a level that often is exceeded in ALF patients.

Microdialysis

Microdialysis is a technique that enables sampling *in vivo* of small hydrophilic molecules in the extracellular compartment of many types of biological tissue. A microdialysis catheter consists of a semi-permeable tip at the end of a biluminal catheter (Figure 3).

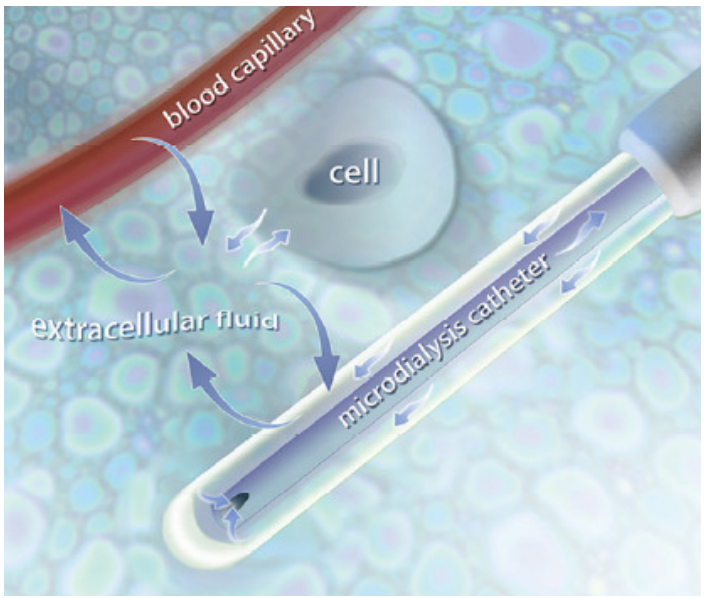

Figure 3 - Schematic drawing of a microdialysis catheter. The perfusate enters the semi-permeable tip through the outer channel where diffusion of metabolites from the surrounding tissue takes place. The perfusate then returns through the inner channel of the catheter and is collected in microvials for later analysis. (Reproduced with permission from CMA).

Microdialysis catheters are made in different sizes suitable for a variety of applications. Ranging from small probes made for animal experimentation in mice and rats to larger probes for clinical use in human tissue for example in liver and brain. By inserting the tip of the microdialysis catheter in the tissue of interest, sampling of the extracellular compartment is made possible by perfusing the catheter with a biochemically neutral aqueous solution, also known as the perfusate. As the perfusate is passing through the semi-membranous tip of the catheter, small hydrophilic molecules in the extracellular compartment will diffuse into the perfusion liquid. The diffusion coefficients of the substances surrounding the microdialysis catheter and the flow rate of the perfusion liquid

determine how close to equilibrium the diffusion processes will reach during the transit time of the catheter tip. The ratio of the concentration of a given substance in the microdialysate and in the surroundings of the catheter is called the recovery ratio. For the most commonly studied substances, such as glucose, lactate, pyruvate and amino acids the recovery ratio is approximately 75-100% with a constant flow of the perfusate at 0.3 µL/min as found in (48, 49) and our own in unpublished in vitro data.

The principle of microdialysis was taken into practice in the 1960s in animal studies which resulted in a small number of publications. Ungerstedt and his colleagues from Stockholm, Sweden, first published clinical results in the late 1980s (50) and during the 1990s publications on the use of microdialysis in human brain tissue emerged (51, 52). Since then, the number of publications has increased almost linearly – and now (July 2011) more than 15.000 publications (both articles and abstracts) are found by searching on the term "microdialysis" on the Web of Science search engine (Figure 4).

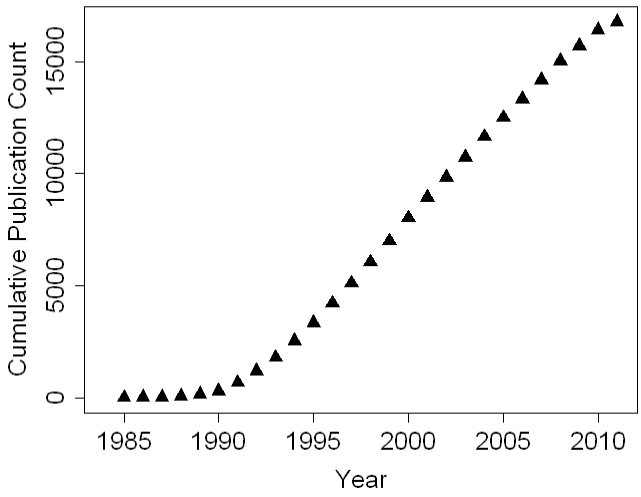

Figure 4 - Cumulative number of publications on microdialysis from 1985 to July 2011.

The use of cerebral microdialysis was introduced on the Department of Hepatology at Rigshospitalet in year 2000. Patients with high risk of cerebral edema that were routinely ICP-monitored also had a microdialysis catheter inserted in the cortical tissue through the same borehole and bolt in the skull as the ICP-catheter. Today microdialysate from 44 patients with ALF has been studied and used both for clinical guidance during the intensive care and for research purposes (Figure 5).

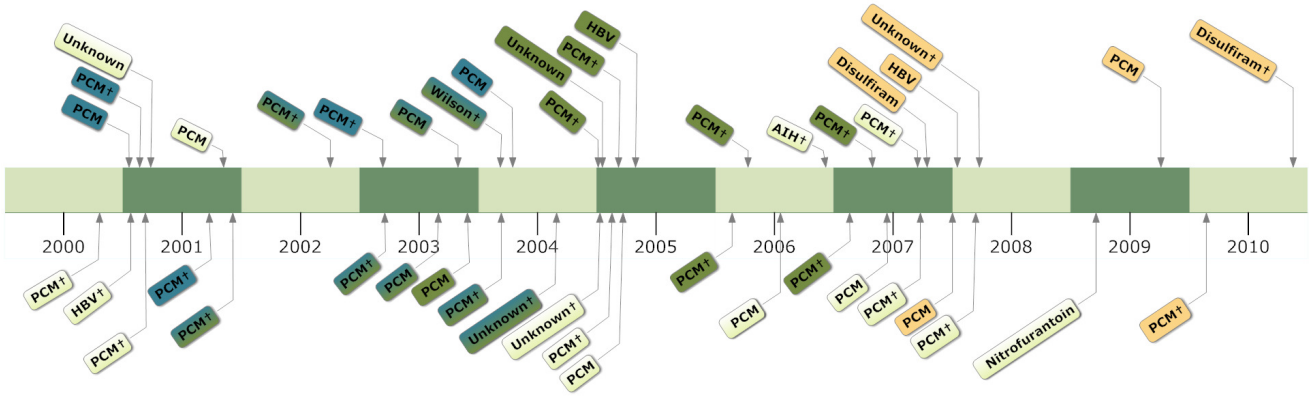

Figure 5 - Timeline of the 44 acute liver failure patients who had cerebral metabolism monitored by microdialysis in the Department of Hepatology since the introduction of microdialysis in 2000. The etiologies of liver failure are stated in the boxes and a '†' denotes death within 30 days after initiation of ICP-monitoring (27/44 patients = 61%). The most frequent etiology of ALF is acetaminophen overdose. AIH: autoimmune hepatitis; Disulfiram: disulfiram hepatotoxicity; HBV: hepatitis B virus infection; PCM: acetaminophen overdose; Wilson: Wilson's disease. The colouring of the boxes indicates which study the patients were included in. Blue: study I; green: study II; yellow: study III.

Fractionated plasma separation and adsorption

Fractionated plasma separation and Adsorption (FPSA) is an extra-corporeal blood purification system marketed by Fresenius Medical Care and used to treat patients with liver failure. The commercial device used for the treatment is named Prometheus®, after the demigod from the Greek mythology.

The Prometheus Myth

According to the Greek mythology, Prometheus gave mankind, which was wild and uncultured at that time, a number of gifts including fire. Zeus became so angry about this, that he chained him to a rock as punishment and an eagle was sent daily to eat parts of his liver so that he should remain sick and weak. Prometheus, however, did not die from this torture

The first publications on FPSA were published more than 10 years ago (53, 54). In 2001 the first clinical FPSA treatment took place in Tübingen, Germany. In 2002 a pilot study was completed and in August 2002 the Prometheus® device became commercially available as a CE marked device. In the Department of Hepatology at Rigshospitalet, the FPSA device has been used since 2004 for selected patients with chronic liver failure.

Although the removal of various toxins by the FPSA system has been verified, to this date no randomised trial has been published that demonstrates reduced mortality. Therefore the FPSA system can only be recommended for protocolled use and no indications are defined for routine use in clinical practice. In the FPSA circuit, the venous blood passes through a plasma separator filter with a pore size of 250 kDa. The resulting filtered plasma phase is then perfused through a column with a neutral resin adsorber (Prometh01) and then a column with an anion exchanger resin adsorber (Prometh02) resulting in removal of albumin bound toxins and negatively charged molecules. The plasma phase is then returned to the plasma filter and is subsequently dialysed as whole blood in a high-flux dialyser (F60S, Fresenius Medical Care AG) leading to removal of water-soluble toxins. Clinical studies have reported significant reductions of creatinine and bilirubin as well as bile acids (55, 56) following therapy. A recent publication demonstrated that a proinflammatory cytokine, tumor necrosis factor alpha (TNF-α), was removed effectively by the system (57) in patients with ALF. The arterial TNF-α has is known to correlate positively with ICP and in patients with uncontrolled ICP a significant efflux of TNF-α from the brain has been described (58).

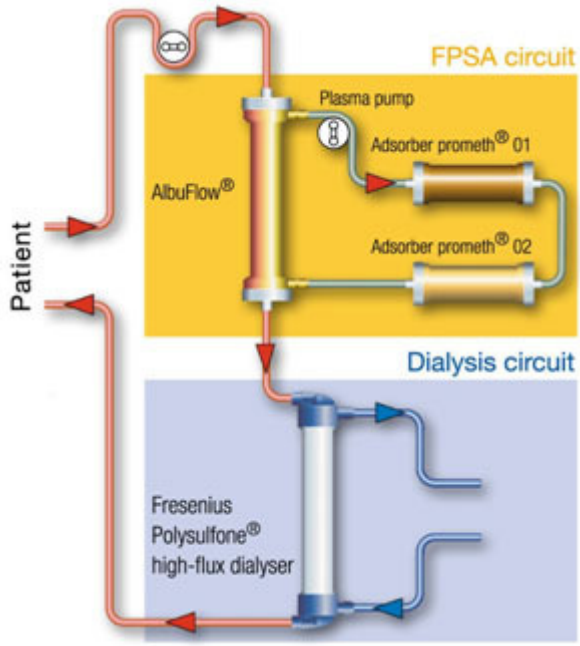

Figure 6 - A schematic drawing of the circuit in the fractionated plasma separation and adsorption (FPSA). Typically a venous catheter is used. The patient's blood first enters the Albuflow filter, which separates plasma from erythrocytes with a cut-off of 250 kDa. The plasma phase is then perfused through the adsorbers Prometh01 and Prometh02 and afterwards returns to the plasma filter. Whole blood is then passing through a high-flux dialyser providing a regular hemodialysis effect. The drawing has been provided by Fresenius Medical Care.

Hypotheses

Ammonia and glutamine has been shown to correlate with ICP and the risk of cerebral herniation in patients with ALF. Studies of cell cultures and laboratory animals have shown results that associate the hyperammonemia and cerebral glutamine accumulation with dysfunction of the mitochondria and a compromised oxidative metabolism of the brain.

The purpose of the studies in this thesis was to test these findings in the clinical setting of ALF, and in addition, to study the effect of intervention with extra-corporeal liver support on cerebral metabolism.

The essential hypotheses are:
In patients with ALF,

- The LP ratio correlates positively to the glutamine concentration in the extracellular compartment of brain tissue.
- The LP ratio correlates positively to ATP breakdown by-products in the extracellular compartment of brain tissue.
- A treatment with the FPSA system reduces cerebral LP ratio, glutamine concentration by reduction of systemic amino acids and the proinflammatory cytokine TNF-α.

Patients and methods

Patients

A total of 29 individual patients with acute liver failure and high risk of cerebral edema were included in the three studies. Eight patients from study I were also included in study II. Acute liver failure was defined according to O'Grady et al (59). Patients were defined as having a high risk of cerebral edema when the arterial ammonia level was above 150 micromolar for more than 24 hours or when clinical signs of cerebral edema were found, such as seizures or abnormal pupillary response. This subgroup of ALF patients routinely had ICP monitored and cerebral microdialysis performed allowing evaluation of brain oxidative metabolism and viability.

Brain microdialysis

The microdialysis catheter was inserted through a borehole in the skull by a neurosurgeon after administration of fresh frozen plasma and activated coagulation factor VII. Following an initialisation period of four to six hours after placement of the microdialysis catheter, sampling of microdialysate was commenced. Samples of varying volume (40-300 microliter) were taken successively while the patients had ICP monitored. The samples were instantly analysed for content of pyruvate and lactate by enzymatic colorimetry (CMA600, CMA, Solna, Sweden) and then frozen for later analysis of amino acids by high-pressure liquid chromatography (HPLC). The sampling interval was determined ad hoc in accordance to clinical events, e.g. microvials would be changed if ICP increased abruptly or at the start of hemodialysis.

HPLC

Amino acid content in microdialysate and in plasma was analysed by ion exchange separation, HPLC with fluorescence detection (Waters HPLC system, Milford, Massachusetts, USA) using a post-column derivatisation. Standard curves were linear within the measured concentrations with the exception of glutamine. Samples were reanalysed, where appropriate, with dilution up to 20 times in order to determine glutamine in all samples.

Ethics

Inclusion in the studies was done after a written consent was obtained from the next of kin. The design of the clinical studies was approved by the Ethical-Scientific Committee (protocols no. H-KF 01-002/00 and H-KF 2007-0006) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Statistics

Correlations were studied by the Pearson product-moment correlation coefficient and Spearman's rank correlation coefficient. Comparison of data was done by either Student’s t-test (paired or unpaired) or Wilcoxon signed-rank test (paired). When data appeared to fit in a normal distribution parametric tests were applied and results expressed as mean ± 1 SD. Otherwise, nonparametric tests were used and results presented as medians (range).

Results

Study I

Brain microdialysate from 13 patients with ALF and high risk of cerebral edema was selected retrospectively and glutamine content and LP ratio was measured. The median glutamine level was 4,396 (range 1,011-9,712) μM and the LP ratio 21.3 (15.8-40.1). We found a strong correlation between glutamine and LP ratio (r^2=0.79, $p < 0.05$) (Figure 7). Additionally, we observed that the ICP but not cerebral perfusion pressure (CPP) correlated to LP ratio (r^2=0.64, $p < 0.05$).

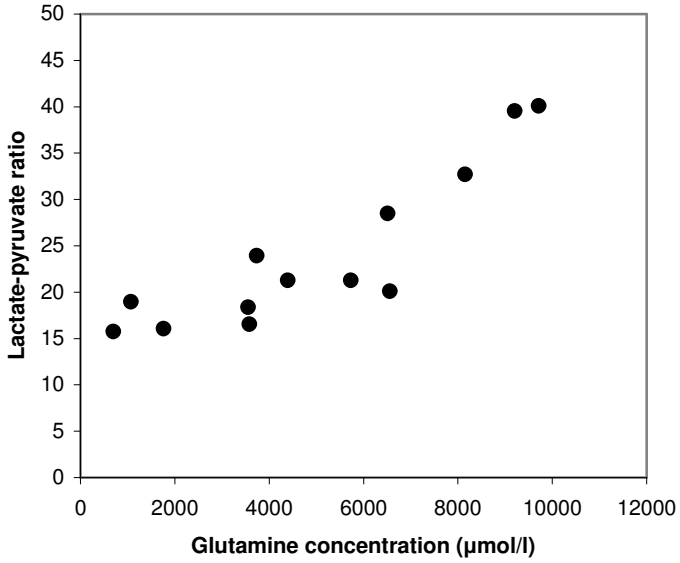

Figure 7 - Scatter plot of LP ratio vs. glutamine concentration. A strong and significant correlation was found (r^2=0.79, $p < 0.05$)

Study II

17 patients with acute liver failure and high risk of cerebral edema were studied. As in study I the microdialysate samples were selected retrospectively. Content of ATP degradation by-products (adenosine, inosine, and hypoxanthine) and the LP ratio was measured. Furthermore, for each patient an early and a late sample were included for analysis of time effect. The cerebral inosine concentration was 7.2 ± 7.1 mM in the early samples and 2.8 ± 1.6 mM in the late samples, while hypoxanthine levels were 23.0 ± 12.0 mM and 11.7 ± 6.8 mM, respectively. The LP ratio (unitless) was 55.6 ± 20.9 and 45.6 ± 20.8 in the early and late samples, respectively. A significant correlation between LP ratio and hypoxanthine was found in both early (Figure 8) and late (Figure 9) samples (r^2=0.38, $p<0.05$ and r^2=0.40, $p<0.01$, respectively). We also observed that the change from early to

late samples in both LP ratio vs. hypoxanthine and inosine also correlated significantly (change in LP ratio vs. change in hypoxanthine: r^2=0.35 and change in LP ratio vs. change in inosine: r^2=0.31, $p<0.05$) indicating that the hypoxanthine and inosine concentrations tend to follow LP ratio over time. We did not find a significant correlation between ICP and hypoxanthine (early samples: r^2=0.12, NS and late samples: r^2=0.15, NS), inosine (early samples: r^2=0.07, NS and late samples: r^2=0.02, NS) or LP ratio (early samples: r^2=0.21, NS and late samples: r^2=0.00, NS). Neither CPP correlated with hypoxanthine (early samples: r^2=0.08, NS and late samples: r^2=0.00, NS), inosine (early samples: r^2=0.07, NS and late samples: r^2=0.02, NS) or LP ratio (early samples: r^2=0.01, NS and late samples: r^2=0.00, NS). The cerebral metabolites did not correlate to the Model of End-stage Liver Disease (MELD) score or the Sequential Organ Failure Assessment (SOFA) score.

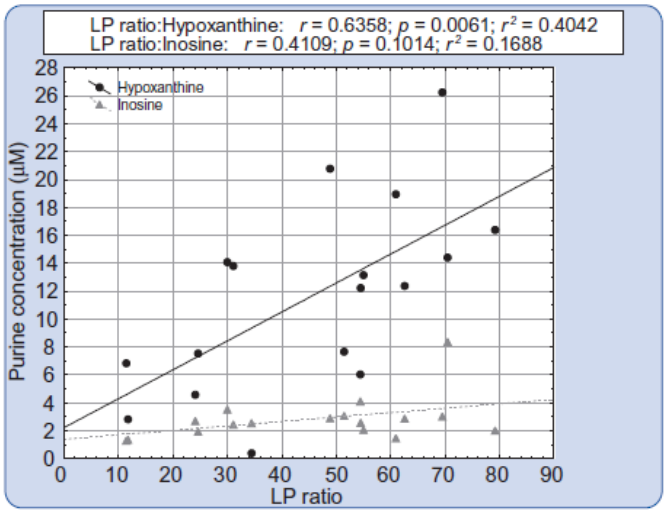

Figure 8 - Scatter plot of hypoxanthine and inosine concentrations against lactate to pyruvate (LP) ratio in early samples with trendlines. A significant correlation was found for hypoxanthine against LP ratio.

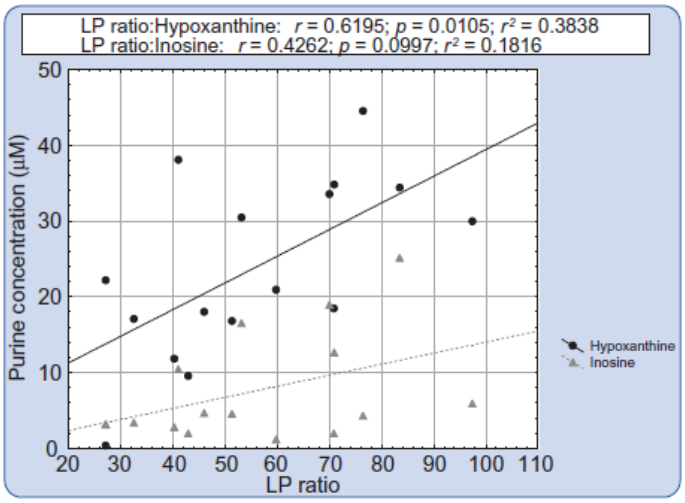

Figure 9 - Scatter plot of hypoxanthine and inosine concentrations against lactate to pyruvate (LP) ratio in late samples with trendlines. A significant correlation was found for hypoxanthine against LP ratio.

Study III

In study III we prospectively included seven patients with ALF and high risk of cerebral edema. Simultaneous samples of arterial blood and venous blood from the jugular bulb as well as microdialysate were obtained before and after a FPSA session. In all samples the concentrations of 19 proteinogenic amino acids were measured by HPLC. In addition we also measured lactate and pyruvate in the microdialysate as well as routine blood tests and TNF-α in the blood samples. The effect of FPSA treatment was modest and we found no changes in the brain concentration of glutamine, LP ratio or oxygen uptake. We did find modest reductions in a few amino acids in brain microdialysate, namely threonine, glutamic acid and histidine (Figure 10). FPSA treatment led to more pronounced changes in the plasma amino acid composition though, most prominent was a decrease in the arterial aromatic amino acid concentration, which led to an increase, but not normalisation, of Fischer's ratio (branched chain amino acids / aromatic amino acids) (Figure 11). We found a small but significant reduction of TNF-α in both arterial and jugular bulb plasma, and a significant reduction of bilirubin (Table 1).

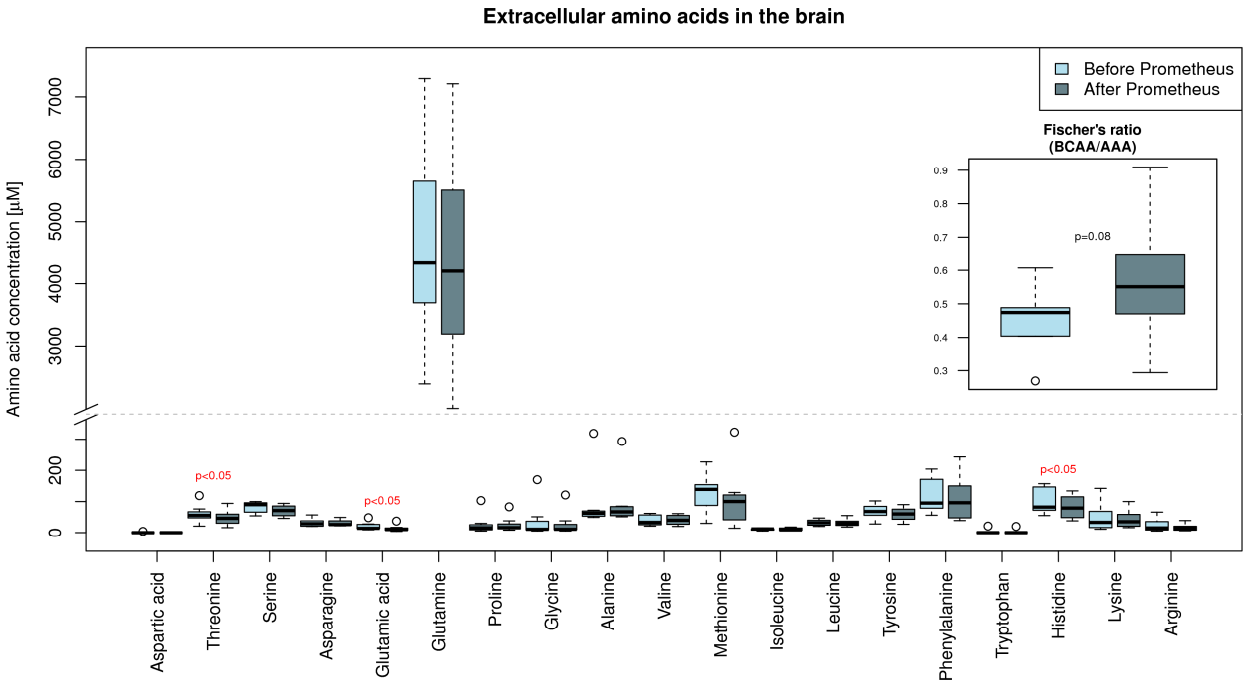

Figure 10 - Box plot of changes in cerebral microdialysate amino acid concentration following FPSA therapy. Significant changes are marked with the p-value of a paired Wilcoxon signed-rank test. BCAA: branched chain amino acids; AAA: aromatic amino acids; FPSA: fractionated plasma separation and adsorption.

Summary of paraclinical parameters before and after FPSA (median (range))

Parameter	Before FPSA	After FPSA	p-value
Body temperature (°C)	35.4 (34.1-37.5)	36.4 (34.0-36.7)	
Bilirubin (µM)	293 (162-487)	249 (144-380)	***<0.05***
Creatinine (µM)	77 (50-152)	51 (37-115)	
Arterial ammonia (µM)	144 (89-225)	115 (40-211)	
Arterial lactate (mM)	3.4 (1.7-7.1)	2.5 (1.1-6.0)	
MAP (mmHg)	75 (67-100)	77 (68-91)	
ICP (mmHg)	9 (3-22)	8 (4-13)	
CPP (mmHg)	67 (59-90)	73 (60-83)	
SOFA score	15 (10-20)	15 (11-20)	
TNF-alpha in arterial plasma (pg/mL)	5.44 (3.95-36.10)	4.30 (2.63-27.99)	***<0.05***
TNF-alpha in jugular bulb plasma (pg/mL)	5.70 (3.56-36.68)	4.24 (3.36-30.28)	***<0.05***

Table 1 - Comparison of levels before and after FPSA was done by paired two-sample Wilcoxon signed rank-test. FPSA: fractionated plasma separation and adsorption. CPP: cerebral perfusion pressure; FPSA: fractionated plasma separation and adsorption; ICP: intracranial pressure; MAP: mean arterial pressure; SOFA: sequential organ failure assessment; TNF: tumor necrosis factor.

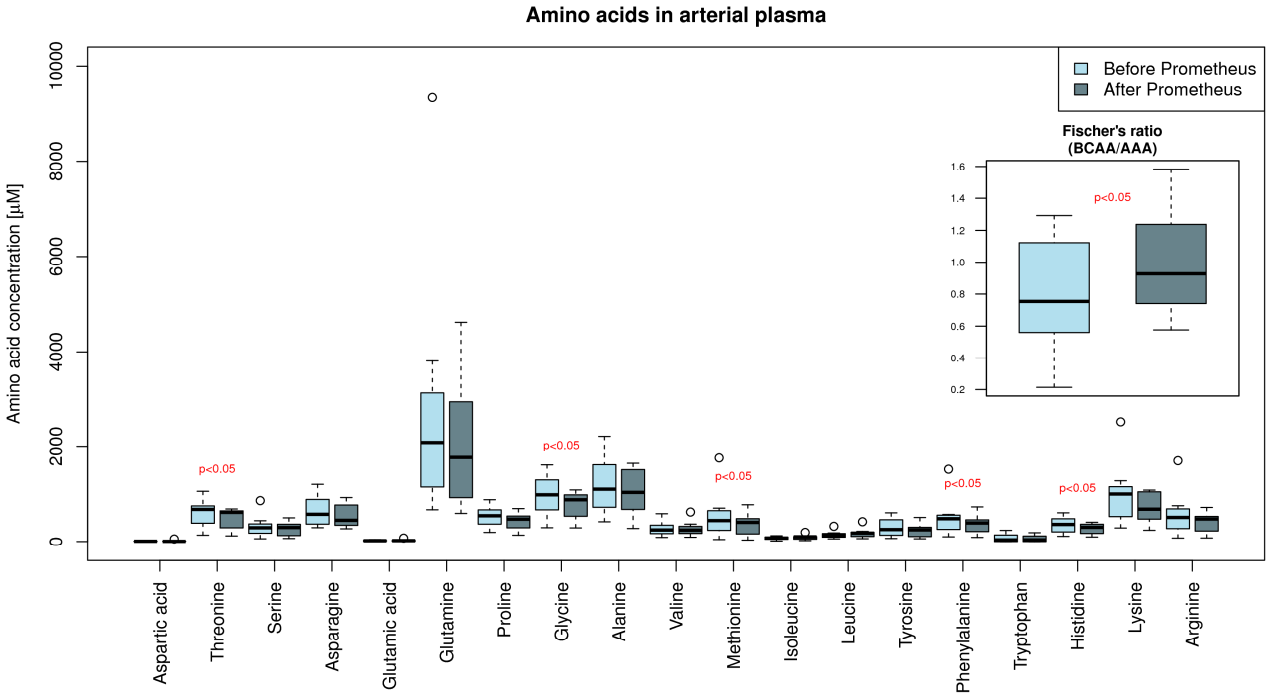

Figure 11- Box plot of changes in arterial amino acid concentrations following FPSA therapy. Significant changes are marked with the p-value of a paired Wilcoxon signed-rank test. BCAA: branched chain amino acids; AAA: aromatic amino acids; FPSA: fractionated plasma separation and adsorption.

Discussion

Study I

We observed that the levels of glutamine in the brain correlated well to the LP ratio in patients with ALF. This is supportive of the hypothesis that oxidative metabolism is compromised by a mitochondrial dysfunction during ALF. ICP also correlated to the LP ratio which is in accordance with the experimental studies showing that ammonia toxicity causes cell swelling by induction of mitochondrial dysfunction, MPT, and nitrosative stress (28, 60, 61). The fact that the lactate-pyruvate ratio was not associated with a low CPP in any of the patients excludes cerebral hypoperfusion as a reason for impaired metabolism.

Study II

A significant correlation between the LP ratio and the ATP degradation products hypoxanthine and inosine was found. This could be explained by multiple modulations of the carbohydrate metabolism such as accelerated glycolysis, inhibition of rate limiting steps in the Krebs' cycle and compromised oxidative phosphorylation in the mitochondrial inner membrane (11, 18, 62). Our modest correlation coefficients indicate that other factors are affecting the extracellular levels of purine derivatives as well. It has previously been demonstrated that acute hyperammonemia leads to accelerated ATP consumption due to activation of NMDA-receptors and Na+/K+-ATPase (63). Also changes in purinergic signaling (e.g. active transport of intracellular ATP and adenosine to the extracellular compartment) could account for some of the variation in hypoxanthine levels.
We found rather high levels of the LP ratios compared to earlier reported reference values found in cortex of patients undergoing surgery for benign cerebral tumors (18 ± 5) (64). Furthermore, the dialysate concentrations of hypoxanthine in our study by far exceeded what has previously been reported in patients with traumatic brain injury (2-6 μM) (45) and in intact rat cortex (1-2 μM) (65)). Although the literature is scarce on reference values for these substances in human brain cortex, our results appear to reflect a highly disturbed metabolism in the brain and resembles the levels seen in cerebral microdialysate of fetal lambs with moderate to severe hypoxia-ischemia (43).

Increased extracellular purine levels traditionally have been associated ischemia (65, 66), but in contrast to this, we here demonstrated an association between high LP ratio and high hypoxanthine

in the absence of hypoxia. This finding could be a result of a metabolic condition described as "cytopathic hypoxia" or "dysoxia", terms used in sepsis literature.

We did not find a significant correlation between ICP and the purine levels or significant differences in brain metabolites by dividing the patients into groups with or without ICH during the study period. In contrast to previously reported findings by our group (67), we did not find a significant correlation between ICP and LP ratio. This indicates that patients with acute liver failure belong to a rather heterogenous group and that the pathogenesis of high ICP is more complex than a simplistic model stating that high arterial ammonia leads to high intracerebral glutamine followed by elevated LP ratio and hypoxanthine, and finally increasing ICP. Additionally, different degrees of low-grade brain edema (68) might have been present in spite of a normal ICP but we did not have an opportunity to measure the water content of the brain. Our hypothesis was moreover challenged by the fact that CPP did not correlate well with hypoxanthine, inosine, or LP ratio. Clearly, other factors such as fluctuations in CBF, systemic inflammation, infections, electrolyte disturbances, and cardiovascular instability must contribute to the development of a disturbed metabolism in the brain and adds to the difficulty of interpreting the influence of the individual parameters on e.g. ICP. In addition to the fact that we did not measure CBF, we did not find it meaningful to perform multivariate analyses due to the limited number of patients in this study.

Finally, it is a noteworthy finding that 9 (53%) of these high-risk patients actually developed high ICP during our study and only one patient of these survived 30 days. Only two patients died directly due to cerebral incarceration but the cerebral edema naturally contributed significantly to the picture of multi-organ failure. In contrast, of the eight patients who did not develop high ICP, four was alive after 30 days.

Study III

We found highly elevated levels of almost all AAs in arterial plasma of patients with ALF. This is in accordance with previous reports (69-72). Only arterial glutamate was lower compared to healthy controls, a finding that might reflect that ammonia detoxification through glutamine synthesis is consuming circulating glutamate. The effect of a single FPSA session on AAs in arterial blood and cerebral microdialysate appeared to be modest with a tendency towards a reduction of most amino

acids, the glutamine concentration was however not affected significantly in neither plasma nor microdialysate. Comparing the significant changes in plasma AAs with the levels of healthy controls the clinical relevance can however be questioned. We were not able to identify a chemical property of the AAs associated with the change in the concentrations. The linear relationship with the arterial concentration is supportive of the fact that unspecific removal is taking place e.g. by passive diffusion in the hemodialysis filter. This was in contrast to our hypothesis, where we expected, for a given AA, that the lower the isoelectric point were, the higher the adsorption in the anion exchanger would be. It is important to note, that the majority of AAs have isoelectric points below the physiological pH in blood and therefore carries a net negative charge in plasma.

We found a significant reduction of the AAAs in arterial plasma, which also accounted for a significant increase of Fischer's ratio. This matches the results from a similar sized study of the effect of FPSA on patients with chronic liver failure (73). Since a low Fischer's ratio is known to be associated with HE (74, 75) it might be a relevant parameter of the treatment effect. Taking into account that Fischer's ratio in healthy subjects is approximately 4.0 we must nevertheless conclude that although we found a significant increase, it must be considered a marginal improvement far from normalisation. To our knowledge the value of Fischer's ratio as a biomarker of HE and brain edema in ALF has not been determined, why our results must be interpreted with some caution.

Although we hypothesized that a tendency towards normalisation of most amino acid would improve cerebral metabolism we did not see any significant effect on the LP ratio in the brain microdialysate. By sampling arterial blood and venous blood from the jugular bulb simultaneously, we were able to measure cerebral oxygen arteriovenous differences. Under the assumption that FPSA does not influence on the cerebral blood flow, our results suggest that FPSA does not alter the oxygen extraction of the brain.

This study was designed to include twelve patients over a three-year study period. However, during the study period it became evident that the incidence of ALF patients with a high risk of intracranial hypertension in our unit was declining. We therefore decided to close the protocol after four years with the inclusion of seven patients. The reduced incidence of intracranial hypertension has also been observed in other centres (76) and it most likely reflects the fact that the management of patients with ALF has improved substantially during the last decade. The incomplete enrolment limits the strength of our conclusions considerably.

Seen as a whole, the effect of a single FPSA session gave very limited impact on the variables studied. This might be partly due to an imbalance between the clearance of the FPSA system and the production rate of cytokines, toxins and amino acids in the patient with ALF. It is indeed possible that longer and repeated FPSA sessions might have had larger impact but it is most often not straightforward to include a 5-hour extra-corporeal blood purification session in the often highly complicated intensive care of ALF patients. It is also possible, that our small sample size is a major reason for our mostly negative results. On the other hand, it is important to emphasize that interventions with a higher 'number-needed-to-treat' might not be reasonable in such volatile clinical conditions with low incidence. Nevertheless, other centres have been able to treat patients with FPSA repetitively for several days and actually achieve significant improvement of variables known to influence on prognosis (77, 78).

In a wider perspective, the current trend in the advances of the handling of ALF patients might, eventually, lead to a therapeutic timeframe large enough for a sufficient number of liver assist sessions to achieve clinically relevant effects. However, large randomised controlled studies of ALF patients are few and complicated to conduct and evidence of effect on hard end-points such as transplant-free survival is difficult to obtain but not impossible (79).

Conclusion and perspectives

The three studies in this thesis include the majority (29 of 44) of patients with ALF and high risk of cerebral edema admitted to our unit the last decade. It is evident that this subgroup of liver failure patients is rare and we have experienced of declining incidence. Nevertheless, we have been able to make firm conclusions in the three studies. In study I we found a strong correlation between high levels of glutamine and high LP ratio. This may be an important result as it is the first human in vivo confirmation of hypotheses based on animal experimentation (80-82). In study II we took a deeper look at the oxidative metabolism of the brain and found that the brain in ALF patients contains high levels of ATP-degradation products which in turn correlate with the LP ratio. This could be a relevant addition to models of brain dysfunction associated with ALF. Again, our study confirmed observations of mitochondrial dysfunction seen in animal models and in vitro models of hyperammonemia (29, 83). Finally, study III, an interventional study, looked at the effect of liver assist therapy on central pathogenic factors in the development of intracranial hypertension during ALF. Although the study was stopped prematurely due to a too slow inclusion rate, we found it safe to conclude that a single treatment session with the FPSA device is not able to radically change the clinical picture of a patient with ALF.

Brain microdialysis in ALF patients is an intervention with a risk of complications, such as intracranial haemorrhage or infection. However, with our procedures this risk appears to be tolerably low. Patients were pretreated with fresh frozen plasma and activated coagulation factor VII. Two of 45 patients have suffered from cerebral complications attributable to a microdialysis catheter. None of them were fatal. Both patients had a hematoma in the tissue surrounding the catheter found by computer tomography (CT). One patient had the hematoma surgically evacuated after liver transplantation and had no cerebral sequelae. With our current knowledge and the limitations in our understanding of how to interpret the extracellular brain metabolites, it is our opinion that cerebral microdialysis in ALF patients only should be performed as part of protocolled studies of patients in whom ICP monitoring is found appropriate.

Seen as a whole, the three studies have a common limitation. Only one or two samples were analysed from each patient and it is difficult to assess causal associations if they are separated over time. For example the maximum glutamine accumulation in brain cortex might precede the increase in LP ratio and hypoxanthine with a substantial interval of hours or days. Likewise, it is our

impression that clinical variables like CPP and ICP have a much more dynamic nature than the concentrations of the cerebral metabolites have. Therefore the correlations found in our studies must be seen with these reservations in mind. Consecutive sampling and recording of clinical parameters would improve the conditions for studying such complex relations. However that was not possible in the retrospective study I and II due to a limited number of samples for each patient. Furthermore, analysing and interpreting very large amounts of data from consecutive samples would be challenging, but can give interesting results as recently reported in a large scale microdialysis study (84).

In conclusion, we have confirmed the hypotheses in study I and II that during ALF, patients with high risk of intracranial hypertension, accumulation of glutamine was associated with impaired oxidative metabolism in the brain. In study III we found that liver assist therapy of the FPSA type did not normalize the metabolic changes in the brain and is probably not able to secure brain viability during ALF complicated with persistent and severe hyperammonemia.

Clinical studies of the pathogenesis of HE in ALF are highly warranted. There is a need to thoroughly study the relevance of the, at times diverging, observations from in vitro studies and animal models of HE. The future handling of ALF patients should address unresolved issues of neuroprotection. Today surges of intracranial hypertension are handled symptomatically and it is essential to define an approach that effectively ensures brain viability during clinical course of these patients in order to improve survival rates. Bioartificial liver support systems have previously suffered from a low functional capacities, but newer systems will most likely achieve clinically relevant clearance rates and synthesis of proteins and hormones (85). Improving the existing regimens used for continuous veno-venous hemodiafiltration might also achieve the goal of removing ammonia from the circulation in a rate that matches the production. This could also be achieved in combination with other modalities, e.g. the promising ammonia scavenger l-ornithine phenylacetate (86). Extracorporeal removal by hemofiltration of proinflammatory cytokines (sepTex^{TM}, Gambro) and also the bacterial endotoxin are available (87). If the capacity of such systems can be validated as clinically relevant they might also contribute to stabilisation of the patients and prevent development of uncontrolled systemic and cerebral inflammation as well as brain edema.

Summary

Acute liver failure is a life threatening disease with a high risk of multi-organ failure. Brain edema with high intracranial pressure is a prominent complication. The mechanism behind brain edema is complex and not fully understood. However, it is known that increasing blood ammonia levels are of central importance. In animal experiments and cell cultures it has been shown that ammonia can disturb both amino acid and glucose metabolism. Moreover, it has been demonstrated that high ammonia levels can lead to a compromised mitochondria function. In this PhD project we studied the brain metabolism of patients with acute liver failure by the use of a microdialysis catheter placed in brain cortex. The purpose of the project was to investigate the association between the brain content of glutamine, the lactate to pyruvate (LP) ratio and adenosine triphosphate (ATP) degradation products. Additionally, we measured the brain metabolism before and after a treatment session with a liver support system, fractionated plasma separation and adsorption (FPSA). We found a significant correlation between the extracellular concentration of glutamine and the LP ratio. Increasing levels of glutamine were associated with increased LP ratio. Likewise we observed a significant correlation between the ATP degradation product hypoxanthine and LP ratio. Both the LP ratio and hypoxanthine levels in the brain were considerably higher compared to studies of other diseases and to normal controls. The effect of FPSA treatment was modest and we found no changes in the brain concentration of glutamine or LP ratio. FPSA treatment led to changes in the plasma amino acid composition though, we the most prominent change was a decrease in the aromatic amino acid concentration. In summary, we concluded that our studies confirmed an association between cerebral accumulation of glutamine and changes in glucose metabolism. The high level of hypoxanthine was compatible with the hypothesis of mitochondria dysfunction. Our results did not indicate that FPSA treatment affects brain metabolism of amino acids and glucose during acute liver failure.

Danish Summary

Akut leversvigt er en livstruende tilstand med høj risiko for multiorgansvigt. En væsentlig komplikation er hjerneødem og deraf følgende højt hjernetryk. Mekanismen bag denne komplikation er kompleks og ikke fuldstændig klarlagt. Det vides dog, at en stigende ammoniumkoncentration i blodet er af væsentlig betydning. I dyreforsøg og i cellekulturer er det vist at ammonium kan forstyrre både sukker og aminosyre-stofskiftet i hjerneceller. Bl.a. er det vist at mitokondriefunktionen nedsættes betydeligt ved høje ammonium koncentrationer. I dette ph.d.-projekt undersøgtes hjernens stofskifte hos patienter med akut leversvigt ved hjælp af et mikrodialyse kateter placeret i hjernebarken. Formålet med projektet var at belyse sammenhænge mellem hjernens indhold af aminosyren glutamin, laktat til pyruvat (LP) ratio og nedbrydningsprodukter af det energibærende adenosintrifosfat (ATP). Endvidere var det formålet at måle stofskiftet i hjernen før og efter en behandlingssession med et levererstatningssystem, fractionated plasma separation and adsorption (FPSA), også kaldet leverdialyse. Vi fandt en signifikant sammenhæng mellem hjernens ekstracellulære glutamin koncentration og LP ratio, idet stigende glutamin koncentrationer var associeret med stigende LP ratio. Ligeledes observerede vi en signifikant sammenhæng med ATP nedbrydningsproduktet hypoxantin og LP ratio. Hypoxantin koncentration og LP ratio var væsentligt forhøjede sammenlignet med studier af andre sygdomme og normalmateriale. Effekten af behandling med FPSA var beskeden og der sås ingen ændringer i hjernens glutamin koncentration eller LP ratio. Behandling med FPSA førte dog til ændringer i plasmasammensætningen af aminosyrer, mest udtalt var faldet i koncentrationen af aromatiske aminosyrer. Samlet set kunne vi konkludere at vores studier bekræfter en sammenhæng mellem ophobning af glutamin i hjernen og påvirkning af sukkerstofskiftet i hjernen. De høje koncentrationer af hypoxantin er forenelige med hypotesen om nedsat mitokondriefunktion. Vi fandt ikke tegn på at behandling med FPSA kan påvirke hjernens metabolisme af aminosyrer og sukker under akut leversvigt.

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Appendices

Appendix I: Bjerring PN, Hauerberg J, Frederiksen HJ, Jorgensen L, Hansen BA, Tofteng F, Larsen FS. Cerebral glutamine concentration and lactate-pyruvate ratio in patients with acute liver failure. Neurocrit Care. 2008;9(1):3-7.